

ENDOTHELIAL COLONY FORMING CELLS (ECFCS): IDENTIFICATION,
SPECIFICATION AND MODULATION IN CARDIOVASCULAR DISEASES

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DEDICATION

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ABSTRACT

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ENDOTHELIAL COLONY FORMING CELLS (ECFCs): IDENTIFICATION, SPECIFICATION AND MODULATION IN CARDIOVASCULAR DISEASES

A hierarchy of endothelial colony forming cells (ECFCs) with different levels of proliferative potential has been identified in human circulating blood and blood vessels. High proliferative potential ECFCs (HPP-ECFCs) display properties (robust proliferative potential in vitro and vessel-forming ability in vivo) consistent with stem/progenitor cells for the endothelial lineage. Corneal endothelial cells (CECs) are different from circulating and resident vascular endothelial cells (ECs). Whereas systemic vascular endothelium slowly proliferates throughout life, CECs fail to proliferate in situ and merely expand in size to accommodate areas of CEC loss due to injury or senescence. However, we have identified an entire hierarchy of ECFC resident in bovine CECs. Thus, this study provides a new conceptual framework for defining corneal endothelial progenitor cell potential. The identification of persistent corneal HPP-ECFCs in adult subjects might contribute to regenerative medicine in corneal transplantation. While human cord blood derived ECFCs are able to form vessels in vivo, it is unknown whether they are committed to an arterial or venous fate. We have demonstrated that human cord blood derived ECFCs heterogeneously express gene transcripts normally restricted to arterial or venous endothelium. They can be induced to display an

arterial gene expression pattern after vascular endothelial growth factor ₁₆₅ (VEGF₁₆₅) or Notch ligand Dll1 (Delta1^{ext-IgG}) stimulation in vitro. However, the in vitro Dll1 primed ECFCs fail to display significant skewing toward arterial EC phenotype and function in vivo upon implantation, suggesting that in vitro priming is not sufficient for in vivo specification. Future studies will determine whether ECFCs are amenable to specification in vivo by altering the properties of the implantation microenvironment. There is emerging evidence suggesting that the concentration of circulating ECFCs is closely related to the adverse progression of cardiovascular disorders. In a pig model of acute myocardial ischemia (AMI), we have demonstrated that AMI rapidly mobilizes ECFCs into the circulation, with a significant shift toward HPP-ECFCs. The exact role of the mobilized HPP-ECFCs in homing and participation in repair of the ischemic tissue remains unknown. In summary, these studies contribute to an improved understanding of ECFCs and suggest several possible therapeutic applications of ECFCs.

Mervin C. Yoder, Jr., MD, Chair

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LIST OF ABBREVIATIONS

AcLDL.....	acetylated low-density lipoprotein
AMI	acute myocardial infarction
Ang	Angiopoietin
APC	allophycocyanin
AV.....	arteriovenous
BAEC.....	bovine aortic endothelial cell
BCAEC	bovine coronary artery endothelial cell
BCEC	bovine corneal endothelial cell
BMP.....	bone morphogenetic proteins
BPAEC	bovine pulmonary artery endothelial cell
BSA	bovine serum albumin
CAC.....	circulating angiogenic cell
CEC.....	circulating endothelial cell
CEC.....	corneal endothelial cell
CFU-Hill.....	colony forming unit-Hill
CNS.....	central nervous system
COUP-TFII.....	chicken ovalbumin upstream promoter transcription factor II
DAB	3,3-diaminobenzidine
DAG.....	diacylglycerol
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
DII1	Delta-like1
DII4	Delta-like4

DPBS.....	Dulbecco's Phosphate Buffered Saline
ECFC.....	endothelial colony forming cell
ECM	extracellular matrix
ECs.....	endothelial cells
EDTA.....	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EPC	endothelial progenitor cell
EPO.....	erythropoietin
ER	endoplasmic reticulum
FACS.....	fluorescence activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FE.....	phycoerythrin
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
Flk-1	fetal liver kinase 1
Flt-1	FMS-like tyrosine kinase 1
Flt-4	FMS-like tyrosine kinase 4
Fn	fibronectin
Foxc.....	Forkhead box c
FSS	fluid shear stress
GrI.....	gridlock

GSL I	<i>Griffonia simplicifolia</i> I lectin
H&E	hematoxylin and eosin
HCP	human umbilical cord plasma
HIF	hypoxia-inducible factor
HPP-ECFC	high proliferative potential-endothelial colony forming cell
HRP	horseradish peroxidase
HUAEC	human umbilical artery endothelial cell
HUVEC	human umbilical vein endothelial cell
IA	intussusceptive angiogenesis
IL6	interleukin 6
IP3	inositol-trisphosphate
ISV	intersegmental vessels
KDR	kinase insert domain receptor
LEL	<i>Lycopersicon Esculentum</i> (Tomato) lectin
LPP-ECFC	low proliferative potential-endothelial colony forming cell
MCP-1	monocyte chemoattractant protein-1
Mib	mindbomb
MMPs	matrix metalloproteinases
MNC	mononuclear cell
MSC	mesenchymal stromal stem cell
NGF	nerve growth factor
NIPs	neuropilin interacting proteins
NK	natural killer

NO	nitric oxide
NOS.....	nitric oxide synthase
Nrp1.....	neuropilin1
Nrp2.....	neuropilin2
PDGFB	platelet-derived growth factor B
PDGFR β	platelet-derived growth factor B receptor β
PDS	plasma derived serum
PECAM1.....	platelet/endothelial cell adhesion molecule-1
PHDs	prolyl hydroxylase domain-containing proteins
PKC	protein kinase C
PLCy.....	phospholipase Cy
PIGF	placental growth factor
PtdIns(4,5)P2.....	phosphatidylinositol-4, 5-bisphosphate
Rbpj	recombination signal binding protein for immunoglobulin kappa J region
S1P.....	sphingosine-1-phosphate
SCF	stem cell factor
SDF1 α	stromal cell derived 1 alpha
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEMA	class 3 semaphorin
sFlt-1	soluble FMS-like tyrosine kinase 1
Shh	sonic hedgehog
Sox	Sry-related HMG box
TGF β	transforming growth factor beta

TM	trabecular meshwork
TNF α	tumor necrosis factor- alpha
TTC	tetrazolium trichloride
UEA I	<i>Ulex europaeus</i> agglutinin I
VE-Cad	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vSMC.....	vascular smooth muscle cells
vWF	von Willebrand factor
Wnt	wingless-int
α SMA.....	smooth muscle alpha actin

CHAPTER I

Introduction

A. The Formation of Functional Blood Vessels

Blood vessels deliver oxygen and nutrients while removing waste from all tissues in the body. The vascular system is hierarchically organized and is composed of functional arteries, capillaries and veins. Capillaries are composed solely of endothelial cells (ECs), and occasionally are ensheathed with pericytes. Arteries and veins have an inner layer of ECs and an outer layer of vessel wall, which consists of the tunica intima, media and adventitia. The formation of blood vessels is an elaborate process including vasculogenesis, angiogenesis, arteriogenesis, vascular remodeling and maturation, all of which involves a wide variety of biochemical and biomechanical factors (Figure I.1).

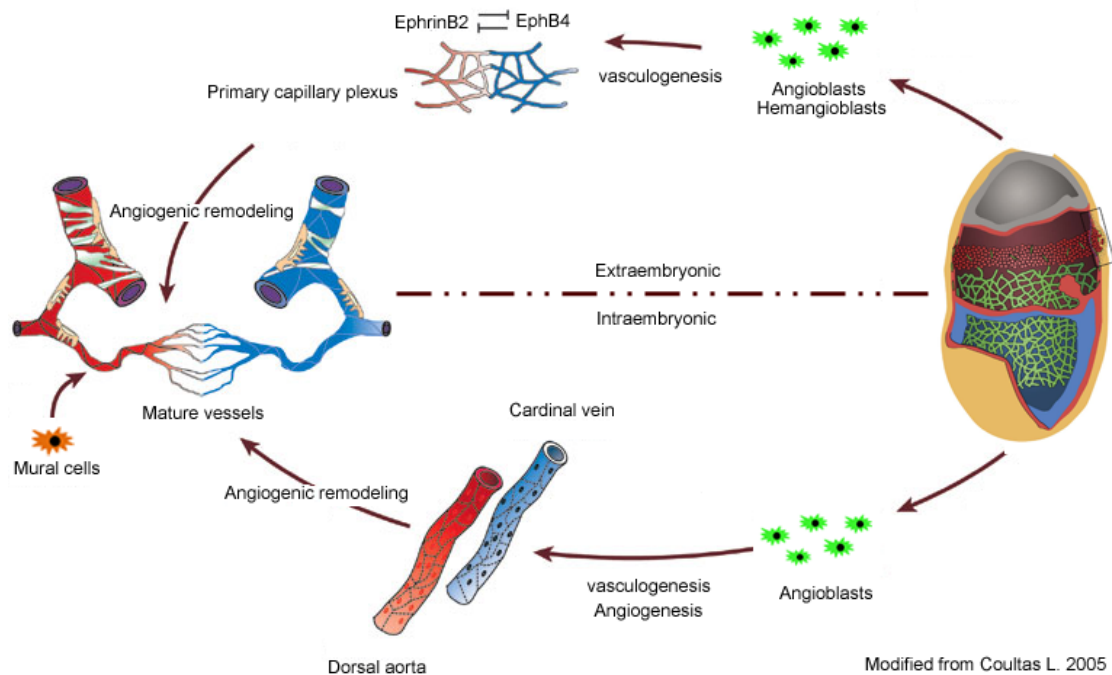


Figure I.1 Formation of a functional vascular network from endothelial precursor cells during murine embryonic development. Mesoderm-derived angioblasts and hemangioblasts give rise to the primitive capillary plexus in the yolk sac via vasculogenesis before circulation is established. Subsequently, the primary capillary plexus quickly expands and undergoes further remodeling. Angioblasts also migrate into the embryo and directly aggregate into dorsal aorta and cardinal vein via vasculogenesis and angiogenesis. These primary vessels later connect to the primary capillary plexus in the yolk sac and form functional vasculature that carries blood.

Vasculogenesis and Regulation

The development of the vascular system is one of the earliest events in embryogenesis. Gastrulation begins at embryonic day (E) 6.5, as evidenced by the formation of the primitive streak and leads to the formation of three principle germ layers: the ectoderm, mesoderm and endoderm (Wells & Melton 1999).

The emergence of endothelial cells (ECs) is first observed in the proximal lateral mesoderm of the extraembryonic yolk sac. The subpopulation of mesoderm cells that expresses vascular endothelial growth factor receptor 2 (VEGFR2, also known as kinase insert domain receptor, KDR in humans; or fetal liver kinase 1 Flk-1 in mice) gives rise to both angioblasts (endothelial progenitors) and hemangioblastic cells (progenitors of both hematopoietic and endothelial lineages) in the yolk sac (Drake & Fleming 2000, Kabrun et al 1997, Kataoka et al 1997, Nishikawa 1997). During the migration of these mesodermal cells, the primary capillary plexus (the earliest blood vessels) begin to form by angioblasts as a honeycomb-like network that separates the distal embryo proper from the proximal blood island region (Ferkowicz & Yoder 2005). At the same time, the earliest hematopoietic progenitor cells (primitive erythrocytes) first appear near the primitive streak and migrate past the developing primitive capillary plexus into the blood island region of the proximal yolk sac, where they rapidly divide and form a circumferential blood cell band at E7.75. The primitive erythrocytes in this region are not surrounded by ECs initially. However, a sheet of ECs exists on the visceral endodermal side of the blood band. ECs appear to invade the blood band, subdividing it into primitive erythrocyte-filled channels that eventually form

a blood-filled vascular bed, which connects to the primitive capillary plexus right before the onset of circulation around E8.25 (Ferkowicz et al 2003, Ferkowicz & Yoder 2005). Extraembryonic EC differentiation initially occurs without concomitant hematopoiesis. Primitive blood vessels are formed *de novo* by the patterned assembly of angioblasts in a process termed vasculogenesis (Risau & Flamme 1995).

Vasculogenesis in the embryo proper appears slightly later and is initiated around E7-7.5 by mesoderm fated to give rise to the endocardium and cranial regions (Drake & Fleming 2000, Tam & Behringer 1997). Subsequently, primary vascular networks appear serially, lateral to the midline, as the paired dorsal aortae, then the head and cardinal vessels (Argraves & Drake 2005, Flamme et al 1997, Lawson et al 2002). Later, neovascularization of heart, lung, liver, spleen and kidney occurs also primarily by vasculogenesis (Aird 2007a, Aird 2007b, Coultas et al 2005, deMello et al 1997, Ferguson et al 2005, Robert et al 1998). In addition, allantoic vasculogenesis occurs by the assembly of mesoderm-derived precursor cells (Downs et al 1998, Drake & Fleming 2000), giving rise to the umbilical vessels (Downs & Harmann 1997). Intraembryonic EC differentiation, similar to that in the yolk sac, initiates in the absence of hematopoiesis.

After the onset of circulation, the primary vascular networks are rapidly expanded and remodeled into arteries, capillaries and veins; ultimately, a functional

circulatory loop is established. As development proceeds, the primitive erythrocytes in circulation are replaced by definitive hematopoietic cells (Brotherton et al 1979, McGrath & Palis 2005, Steiner & Vogel 1973). Thus, vasculogenesis collectively results in the formation of the primary capillary plexus in the yolk sac and the major embryonic vessels.

Mouse knockout studies have identified many genes that contribute to vasculogenesis (Argraves & Drake 2005). While several signaling pathways participate into this process, including FGF signaling (Poole et al 2001, Smith 1989), Wnt signaling (Wang & Wynshaw-Boris 2004, Zerlin et al 2008), BMP signaling (Winnier et al 1995) TGF β signaling (Sirard et al 1998, Yang et al 1998) and Indian hedgehog signaling (Dyer et al 2001), none of these signaling cascades is specific for developing the endothelial lineage.

In sharp contrast, Flk-1 plays a vital role in differentiating mesoderm exclusively to endothelial and hematopoietic lineages in the early gastrulating embryo (Dumont et al 1995, Roman & Weinstein 2000). Flk-1 deficiency (Flk-1^{-/-}) severely impairs the development of ECs and hematopoietic cells, and causes embryonic death at E8.5 (Shalaby et al 1995). Similarly, vascular endothelial growth factor (VEGF, Flk-1 ligand) is indispensable for vasculogenesis. Targeted deletion of VEGF leads to embryonic lethality. VEGF^{-/-} embryos display a phenotype similar to that observed in Flk-1 knockout mice, but considerably less severe (Carmeliet et al 1996, Ferrara et al 1996). Interestingly, further studies

demonstrate that VEGF is haploid-insufficient — loss of a single VEGF allele is lethal in the mouse embryo, causing abnormal blood vessel development. This indicates a tight dose-dependent regulation of embryonic vessel development by VEGF.

Vascular endothelial growth factor receptor 1 (VEGFR1 or FMS-like tyrosine kinase 1, Flt-1) is another VEGF receptor and is also primarily expressed in ECs. Flt-1 knockout mice exhibit normal EC differentiation but abnormal vascular organization in early embryogenesis (Fong et al 1995, Fong et al 1999). Flt-1 has a higher affinity for VEGF binding but lower kinase activity compared to Flk-1. Thus, Flt-1 might serve in part to modulate Flk-1 activity by sequestering excess VEGF stimulation.

Angiogenesis and Regulation

Once the basic pattern of primitive extraembryonic and intraembryonic vasculature is formed by vasculogenesis, blood vessels are expanded and remodeled rapidly. This process, referred to angiogenesis, is defined as the expansion of blood vessels from preexisting vessels. Angiogenesis involves many morphological events including sprouting EC growth, intussusceptive growth, stabilization, remodeling, pruning and specialization (Carmeliet 2000, Flamme et al 1997, Jain 2003, Patan 2004, Risau & Flamme 1995).

Sprouting angiogenesis happens in many regions such as the yolk sac, central nervous system (CNS) and retina (Breier & Risau 1996, Gerhardt et al 2003, Kurz et al 1996, Noguera-Troise et al 2006, Ridgway et al 2006). Angiogenic sprouting is characterized by leading tip cells and trailing stalk cells (Gerhardt et al 2003). This process could be prompted by an insufficient supply of oxygen and guided by VEGF via Notch signaling (comprehensively reviewed in Gerhardt 2008 and Phng 2009) (Gerhardt 2008, Phng & Gerhardt 2009). Hypoxia upregulates VEGF expression and activates nitric oxide synthase (NOS) which leads to the bulk production of nitric oxide (NO) (Fraisl et al 2009). Existing vessels then dilate in response to NO and become leaky in response to VEGF. With the involvement of active proteases, the basement membrane and extracellular matrix (ECM) are degraded. These events make ECs acquire invasive and migratory ability.

ECs stimulated by VEGF compete for the tip cell position via Dll4/Notch1 signaling. The cell that produces more Dll4 than its neighbors eventually will remain as a tip cell because it can sufficiently suppress the same response in adjacent ECs via activated Notch1 signaling (Phng & Gerhardt 2009). VEGF also induces the expression of its receptor Flk-1 and the formation of long, dynamic filopodia in the tip cells. Thus, tip cells are able to migrate along VEGF gradients. The VEGF gradient on EC surface is tightly regulated by mRNA levels, isoform splicing, cell membrane retention and probably protein degradation. A spatial concentration gradient of membrane-bound VEGF (VEGF₁₈₈ and VEGF₁₆₄ in

mice, and VEGF189 and VEGF165 in humans) functions as a chemoattractant signal that promotes the polarized extension of tip cells. In contrast, diffused VEGF (VEGF120 in mice; VEGF121 in humans) promotes EC proliferation but does not guide tip cells (Gerhardt 2008).

While sprouting, the vascular lumen can form by ECs in the absence of mural cells. Lumenization requires the coordinated participation of multiple molecules including small GTPases and cell-cell, cell-matrix adhesion proteins (Avraamides et al 2008, Dejana et al 2009, Horowitz & Simons 2008, Iruela-Arispe & Davis 2009, Koh et al 2008). Furthermore, growing vascular sprouts can generate gradients of platelet-derived growth factor B (PDGFB), which promote the recruitment of pericytes via PDGFB receptor β (PDGFR β). This ensures that the growing endothelium is efficiently stabilized by mural cells, which enable ECs to withstand the physical forces of blood flow (Gerhardt et al 2003).

Each new spout ultimately needs to suppress its motile behavior and connect with adjacent sprouts or existing capillaries. Flow-dependent tissue oxygenation downregulates paracrine VEGF production and thus helps establish a quiescent state for these new vessels. The investment of mural cells and the deposition of ECM proteins into the subendothelial basement further contribute to vessel quiescence and maturation (Jain 2003, Jones et al 2006, Lucitti et al 2007). The basement membrane provides critical support for the endothelium and fundamentally affects its status, mainly through adhesive interaction with

integrins on the surface of ECs. For example, laminin-binding integrins such as $\alpha 3\beta 1$ and $\alpha 6\beta 1$, as well as collagen IV and perlecan, are important for regulating tube stabilization and EC quiescence (Davis & Senger 2005, Iruela-Arispe & Davis 2009, Stratman et al 2009). Moreover, plasmin and Matrix metalloproteinases (MMPs) (such as MMP3) are reported to be able to release VEGF₁₆₅ from the ECM by converting it into a soluble isoform VEGF₁₂₁-like component and sequester VEGF₁₆₅ signaling, restoring EC quiescence (Mancuso et al 2006). Furthermore, vascular architecture specializes according to local tissue and organ growth, such as the formation of heart valves and fenestration. Lastly, arteries and veins form and expand, acquiring additional layers of mural cells, ECM and elastic laminae coverage, giving the vessels full ability to sense pressure and respond accordingly.

Cumulative evidence indicates that many elements are capable of influencing angiogenesis, including oxygen, metabolic intermediates, blood flow, cytokines and growth factors (Adams & Alitalo 2007, Fraisl et al 2009, Jain 2003, Larrivee et al 2009). The principal player in angiogenesis is VEGF, which promotes EC sprouting, proliferation, migration, differentiation and survival, and controls EC-EC, EC-ECM interactions and more. Many other molecules such as fibroblast growth factor (FGF), Angiopoietin/Tie2, Notch, TGF β /Alk5, S1P/Edg1, Ephrin/Eph and integrins also participate in regulating angiogenic responses.

VEGF and VEGF receptors

The VEGF family belongs to the platelet-derived growth factor (PDGF)/VEGF supergene family. At least 7 members comprise this family: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF) and snake venom-derived VEGFs. NOTE: In this chapter, “VEGF” stands for VEGF-A. Binding of VEGF to KDR is the main extracellular signal triggering angiogenic responses, for example, regulating EC proliferation and NO generation. KDR efficiently activates phospholipase C γ (PLC γ), which cleaves phosphatidylinositol-4, 5-bisphosphate (PtdIns(4,5)P₂) to produce diacylglycerol (DAG) and inositol-trisphosphate (IP₃). These soluble products release calcium from the endoplasmic reticulum (ER) and activate protein kinase C (PKC). The latter activates MRK-MAPK pathway which affects DNA synthesis in ECs (Takahashi et al 1999, Xia et al 1996). In addition, VEGF increases eNOS activity through a pathway mediated by Src and thus produces NO. Calcium release also helps the production of NO. Thereafter, VEGF via NO affects EC division, migration and apoptosis (Donnini & Ziche 2002, He et al 1999). Moreover, VEGF via activated Notch signaling can regulate many steps of angiogenesis, of which endothelial sprouting and arterial-venous specification have been investigated at length (Ahmed & Bicknell 2009, Coultas et al 2005, Gerhardt 2008, Holderfield & Hughes 2008, Roca & Adams 2007, Siekmann et al 2008).

VEGF can interact with both VEGF receptors Flt-1 and Flk-1. Flt-1 has weak tyrosine kinase activity and high affinity for VEGF, which makes it act as a decoy

receptor, modulating angiogenesis through its ability to sequester VEGF and thereby reduce signaling through Flk-1 (Park et al 1994). Consistently, deletion of Flt-1 gene in the embryo or embryonic stem-cell-derived vessels induces overgrowth of endothelial cells and vessel dysmorphogenesis (Fong et al 1995, Fong et al 1999). By contrast, mice that express a membrane-anchored Flt-1 variant lacking a tyrosine-kinase domain (Flt-1TK^{-/-}) but still capable of binding VEGF are basically healthy and do not exhibit vascular defects (Hiratsuka et al 1998). It is of interest that the Flt-1 gene also encodes a secreted soluble protein that has only one ligand-binding region, called soluble Flt-1 (sFlt-1) (Kendall & Thomas 1993, Shibuya et al 1990). sFlt-1 level was reported to be abnormally elevated in the serum of preeclampsia patients. Recent studies indicate that abnormal trapping of VEGF with excess sFlt-1 causes hypertension and proteinuria, which are the major symptoms of preeclampsia (Koga et al 2003, Maynard et al 2003). Similarly, overexpression of sFlt-1 induces over-proliferation of glomerular ECs with the loss of endothelial fenestration (a hallmark of the glomerular vascular endothelium) that resembles the renal histological lesions of preeclampsia (Baumwell & Karumanchi 2007). Moreover, sFlt-1 has recently been indicated to play an important role in angiogenic sprouting (Chappell et al 2009, Kappas et al 2008). Spatially regulated expression of sFlt-1 in conjunction with VEGF contributes to the formation of spatial VEGF gradient and therefore guides emerging sprouts away from parent vessels. Thus, Flt-1 regulates vascular development by sequestering excess VEGF and contributes to maintain vascular homeostasis.

Flt4 is expressed mainly in lymphatic ECs and through response to VEGF-C and VEGF-D regulating lymphangiogenesis. However, Flt4 also plays an important role in VEGF signaling in angiogenesis (Dumont et al 1993), likely by forming a heterodimer with KDR (Dixelius et al 2003). Flt4 abundant expression has been observed in zebrafish intersegmental vessels (ISV), at the tip cells of ISVs in mouse embryos and in the front of sprouts in mouse retinas (Gerhardt et al 2003, Siekmann & Lawson 2007). Interestingly, loss of Notch signaling increases Flt4 expression in stalk cells, while suppression of Flt4 expression partly restores the sprouting, indicating that Flt4 is downregulated by Notch activity in stalk cells. However, one in vitro study suggested activated Notch signaling can upregulate Flt4 (Shawber et al 2007). Therefore, the underlying mechanism needs to be clarified.

Neuropilins (Nrps) including Nrp1 and Nrp2 play an important role in both neuronal and blood vessel development. They are receptors for two types of ligands, the class 3 semaphorin (SEMA) family of axon guidance molecules and the VEGF family of angiogenic factors. Nrp1 is primarily expressed in arteries while Nrp2 is abundant in veins and lymphatic vessels (Herzog et al 2001, Yuan et al 2002). The first evidence of Nrp1's involvement in angiogenesis was that overexpression of Nrp1 in transgenic mice resulted in embryonic lethality and vascular defects; for example, excess numbers of blood vessels and hemorrhage. (Kitsukawa et al 1995). Nrp1-deficient mice exhibit abnormal vascular development, including impaired neural vascularization and

disorganized, insufficient development of vascular networks in the yolk sac and the mice die *in utero* (Kawasaki et al 1999). However, Nrp2-deficient mice are viable; they develop arteries and veins normally but fail to develop small-diameter lymphatic vessels and capillaries (Yuan et al 2002). In addition, Nrp1/Nrp2 double-knockout mice display more severe vascular defects than Nrp1-deficient mice and die at E8.5 (Takashima et al 2002). Recent studies indicate that Nrp signaling can be independent of VEGF/KDR, by binding Nrp interacting proteins (NIPs) such as RGS-GAIP Interacting Protein (GIPC) and Synectin (Cai & Reed 1999, Chittenden et al 2006, Gao et al 2000). Collectively, the roles of Nrps in angiogenesis need to be clarified in greater depth.

Angiopoietins and Tie receptors

The angiopoietin family is composed of four ligands (Angiopoietin1, Angiopoietin2 and Angiopoietin3/4) and two corresponding tyrosine kinase receptors (Tie1 and Tie2). Ang3 and Ang4 are orthologs found in mice and humans, respectively. Tie receptors are specifically expressed in the endothelium (Thomas & Augustin 2009, Yancopoulos et al 1998). Tie2 is the receptor for all four angiopoietin ligands, but the ligand for Tie1 is not clear yet. It recently has been shown that Tie1 can interact with Tie2 to form a heteromerized signaling complex (Saharinen et al 2005). Tie1-deficient mice either die *in utero* (Puri et al 1995) or perinatally (Sato & Rifkin 1989) with edema and hemorrhage, which indicates Tie1 is associated with angiogenesis.

Ang1 is expressed mainly in mural cells (Ramsauer & D'Amore 2002), but Ang2 is produced and stored in ECs (Eklund & Olsen 2006, Fiedler et al 2006). The Ang1/Tie2- dependent signaling cascade has been demonstrated to promote angiogenesis, stabilize nascent vessels via recruitment of pericytes, reduce vascular permeability and exhibit anti-inflammatory activity (Armulik et al 2005, Eklund & Olsen 2006, Koblizek et al 1998, Thurston et al 1999). Mice embryos lacking Tie2 and Ang1 initially develop a rather normal vascular network, but it fails to be further remodeled, which is similar to mice in which Ang2 is overexpressed in the endothelium (Dumont et al 1994, Maisonpierre et al 1997, Sato et al 1995, Suri et al 1996). This observation indicates that Ang2 may counteract Ang1 signaling. Further studies reveal that the role of Ang2 is contextual. In the absence of VEGF, Ang2 destabilizes vessels and contributes to vascular regression; but in the presence of VEGF, Ang2 becomes angiogenic and facilitates vascular sprouting (Eklund & Olsen 2006, Maisonpierre et al 1997).

FGF and FGF receptors

The FGF family members are heparin-binding protein mitogens that play critical roles in diverse biological processes (Itoh 2007). This family is composed of 22 FGF ligands and four tyrosine kinase receptors (Eswarakumar et al 2005). While FGFs promote a strong angiogenic response, FGF-induced angiogenesis appears to require activation of VEGF signaling (Presta et al 2005). Several lines of evidence suggest that FGF regulates both VEGF and KDR expression in ECs.

However, once VEGF signaling is activated, its ability to induce angiogenesis appears to be independent of FGF signaling (Murakami & Simons 2008).

Moreover, mice that are null in FGFR1, FGFR2, FGFR3, FGFR4, FGF1, FGF2 and FGF1/FGF2 don't exhibit abnormal vascular defects (Arman et al 1998, Deng et al 1994, Miller et al 2000, Ortega et al 1998, Weinstein et al 1998, Yamaguchi et al 1994, Zhou et al 1998). These findings imply extensive redundancy of FGFs and leave their distinct role in vascular development unclear.

PDGF and PDGF receptors

PDGFs and their receptors (PDGFRs) have long served as prototypes for growth factor and receptor tyrosine kinase function. The PDGF family consists of PDGFA, PDGFB, PDGFC and PDGFD, encoded by genes PDGF-A and PDGF-B (Heldin & Westermark 1999). PDGF receptors contain PDGFR α and PDGFR β . PDGFR β is expressed in microvascular ECs in vitro and contributes to EC proliferation, sprouting and lumen formation (Bar et al 1989, Battegay et al 1994). Moreover, the expression of PDGFR β in pericytes and vascular smooth muscle cells (vSMCs) is very critical for mural cell proliferation, guided migration and incorporation into the vessel wall. The expression of PDGFB in nascent vessels favors recruitment of mural cells that expressing PDGFR β (Armulik et al 2005, Betsholtz et al 2005).

PDGFR β function may involve cooperation with a family of G-protein coupled receptors that bind to sphingosine-1-phosphate (S1P), called S1P receptors (S1PR1-5) (Allende & Proia 2002, Spiegel & Milstien 2003). S1PR1-deficient mice exhibit a phenotype similar to that of PDGFB- and PDGFR β -knockout mice, where mural cells fail to migrate to blood vessels (Kono et al 2004). S1PR1 is expressed mainly in ECs and involves trafficking of N-cadherin to the endothelial-mural-cell contact region (Allende et al 2003, Paik et al 2004). Endothelial N-cadherin is important for the expression of adhesion molecules, embryonic survival and cardiovascular development (Resink et al 2009). Therefore, PDGFB-PDGFR β signaling affects vascular homeostasis.

Oxygen regulation

Oxygen tension can determine whether blood vessels maintain quiescence or undergo angiogenesis. A major link between hypoxia and angiogenesis is the upregulation of hypoxia-inducible factor (HIF), which plays a central role in the transcriptional activation of angiogenic factors. HIFs are heterodimeric transcriptional factors consisting of α and β subunits. While HIF1 β is insensitive to oxygen, HIF1 α and HIF2 α are oxygen-sensitive and are rapidly degraded by catalytic hydroxylation of prolyl hydroxylase domain-containing proteins (PHDs). In general, HIF1 α is expressed ubiquitously (Semenza 2003), but HIF2 α is expressed primarily in the endothelium. During hypoxia, HIFs can induce gene expression via direct binding to hypoxia response elements (HRE) on gene promoters. These genes include VEGF (Forsythe et al 1996), Flt-1 (Gerber et al

1997, Takeda et al 2004), erythropoietin (EPO) (Morita et al 2003, Semenza & Wang 1992), and eNOS (Coulet et al 2003). Also, many genes not known to contain HRE still can be activated by HIF, such as FGF2, PlGF, PDGFB, Ang1, Ang2 and Tie2. Hypoxia induces VEGF expression in ECs and perivascular cells and regulates EC functions via autocrine and paracrine VEGF signaling. It is very interesting to note that intracellular VEGF/KDR signaling plays an important role in maintaining EC viability and vascular integrity, which is supported by the observation that EC-specific deletion of VEGF leads to EC apoptosis and loss of vascular integrity (Lee et al 2007).

Genetic modification experiments have revealed the distinct roles of HIFs in vascular development. HIF1 β knockout mice display deficient angiogenesis in the yolk sac, which is embryonically lethal (Maltepe et al 1997). HIF1 α -deficient mice exhibit severe vascular defects (Carmeliet et al 1998, Ryan et al 1998) that cannot be rescued by HIF2 α . This indicates the functions of HIF1 α and HIF2 α are distinct, rather than overlapping. On the other hand, overexpression of HIF1 α promotes revascularization, improving perfusion in ischemic tissues in rabbit models with hindlimb ischemia (Vincent et al 2000). Targeted deletion of HIF2 α also causes many vascular abnormalities, according to different genetic backgrounds (Compernelle et al 2002, Duan et al 2005, Scortegagna et al 2003). Moreover, the lack of both HIF1 α and HIF2 α leads to impaired vascular remodeling and failure of vascular sprouting in the embryo and yolk sac (Licht et al 2006). In addition, HIF2 α is uniquely able to induce the expression of eNOS

and VE-Cad, which affects vascular remodeling and maturation (Coulet et al 2003, Le Bras et al 2007).

Theoretically, PHD activity could regulate angiogenesis. PHD2-null embryos die *in utero* but do not display increased angiogenesis, although HIF1 α and HIF2 α levels are elevated significantly (Aragones et al 2008, Takeda et al 2007). One possible explanation is that angiogenesis in the developing embryo is intact during this time window and excess HIF- α accumulation has a relatively insignificant impact. In contrast, PHD2 deficiency in adult mice results in significantly increased angiogenesis (Haase et al 2001, Rankin et al 2005, Takeda et al 2007). Furthermore, PHD1- and PHD3-knockout embryos are apparently normal, which might be due to compensation by PHD2, because the latter is the most abundantly expressed PHD isoform. Therefore, PHDs are important for maintaining vascular integrity in the adult. Collectively, the regulation of angiogenesis by oxygen tension is a complex process involving numerous molecular mechanisms and requires further in-depth investigation.

Integrins

The ECM serves as a storehouse for various growth factors and proenzymes that regulate angiogenesis. ECs adhere to the ECM through the expression of surface-bound integrins. Integrins have 18 unique α and 8 unique β subunits and at least 24 distinct α/β integrin heterodimers have been identified. Integrins interact with many basement membrane components, such as fibronectin (FN),

vitronectin, collagen, laminin and heparin-sulfate proteoglycans, to form focal adhesion complexes and thus regulate EC proliferation, migration, adhesion and survival (Cheresh & Stupack 2008).

The proteolysis of many basement membrane proteins produces many antiangiogenic fragments including angiostatin, endostatin, kininostatin, endorepellin, restin, tumstatin and vastatin (Chen et al 2006, Jimenez et al 2000, John et al 2005, O'Reilly et al 1997) and the molecular mechanisms by which they function are yet unknown. It is likely that many as yet uncharacterized fragments remain to be discovered. Endorepellin, a C-terminal proteolytic fragment of perlecan, is one example of our incomplete knowledge of its functionality (Bix et al 2004). Endorepellin binds to integrin $\alpha 2\beta 1$ (a collagen receptor), resulting in EC actin cytoskeleton disassembly and focal contact disruption, causing migration failure and aborted tube formation. Interestingly, endorepellin also can bind to endostatin and counteract its antiangiogenic activity. In addition, many of these proteolytic fragments require circulating forms of plasma FN and vitronectin for their antiangiogenic activities (Akerman et al 2005). Thus, the precise roles of the interaction between integrins and basement membrane in angiogenic regulation need further investigation.

Intussusceptive Angiogenesis (IA) and Regulation

Non-sprouting angiogenesis or IA is a process in which transvascular tissue pillars form within capillaries, small arteries, and veins and subsequently fuse.

This results in the formation of vascular trees (intussusceptive arborization) or vessel remodeling (intussusceptive branching remodeling) (Burri et al 2004, Makanya et al 2009). It was first observed in developing pulmonary vessels (Patan et al 1993) and later was found in other organs and tissues such as the heart and yolk sac during embryonic development. IA's direct influence on structural remodeling optimizes vessel formation and function in the local organ (Kurz et al 2003). Although hemodynamic forces such as increased blood flow have been demonstrated to directly influence IA to initiate pillar formation, the underlying molecular mechanism is still unclear. Moreover, little is known about the molecular regulation of IA.

Arteriogenesis and Regulation

As discussed already, sprouting angiogenesis leads to an increase in capillary vessel density and improves blood perfusion of hypoxic tissue. Thus, sprouting angiogenesis is necessary to maintain or restore local oxygen and nutrition supplies and prevent vessel stenosis. However, this process may not be sufficient to restore the function of larger arteries (Scholz et al 2002). In contrast, arteriogenesis—defined as the growth of functional collateral arteries from preexisting arterio-arteriolar anastomoses (Schaper & Schaper 1997)—partially contributes to the rescue of artery function. Arteriogenesis is initiated by altered fluid shear stress (FSS) caused by a blood flow change due to an arterial occlusion and is independent of hypoxia (Deindl et al 2001, Ito et al 1997).

Numerous studies in experimental animal models with hindlimb ischemia indicate arteriogenesis is a complicated process that relies on the interaction of FSS, growth factors and cytokines, proteolytic enzymes and local inflammation. Arteriogenesis can be divided roughly into four phases (Schaper 2009). The first phase follows an initial artery occlusion. During this phase, quiescent ECs and vascular smooth muscle cells (vSMCs) become proliferative and vascular permeability is increased. The second phase is characterized by destruction of the collateral artery by degradation of the basement membrane, internal elastic lamina and collagen, accompanied by a burst of proliferation in ECs and vSMCs. Thereafter, the maturation phase proceeds and can be described as the orderly arrangement of vSMCs in multiple layers, the synthesis of elastin and collagen to generate new extracellular scaffolds, and the reestablishment of cell-cell and cell-matrix association. At the last phase, pruning and remodeling occur; only a few large vessels continue to grow, while great numbers of small vessels regress by the competition for blood flow.

Although arteriogenesis has been well illustrated, its precise underlying molecular mechanisms remain elusive and need further investigation. ECs sense changes in FSS, which leads to activation of eNOS. Then eNOS generates NO, which induces VEGF expression. Upregulated VEGF in ECs stimulates Monocyte chemoattractant protein-1(MCP-1) expression in vSMCs, which triggers activation, migration and adhesion of monocytes to the endothelium. After monocytes mature into macrophages, these cells produce additional

cytokines (such as FGF and TNF α) that contribute to vSMC proliferation. They also produce proteases (such as MMPs), which help digest the basement membrane. Consequently, vSMCs lose their tight cell-cell and cell-matrix connections, allowing vessels to enlarge (Cai et al 2000). Moreover, the interaction between PDGFB (expressed by ECs) and PDGFR β (present on vSMCs) contributes to the migration and recruitment of vSMCs to ECs where they form the neointima layer (Hellstrom et al 1999). Additionally, vSMCs are involved in reconstitution of elastin lamina and the tunica media. The active status of vSMCs is finally shut down when vessel maturation is complete (Scholz et al 2000).

Besides monocytes, T cells and NK cells recently were reported to be involved in arteriogenesis; but their roles are unclear (Heil & Schaper 2004, Stabile et al 2003, van Weel et al 2007). Bone marrow-derived cells also have been described to be recruited to the growing collateral artery, but they do not incorporate into the vessel wall (Kinnaird et al 2004, Ziegelhoeffer et al 2004). This suggests that paracrine signaling of cytokines or growth factors produced by these cells contributes to arteriogenesis. This makes bone marrow-derived cells and growth factors as interesting targets for clinical therapeutics (van Oostrom et al 2008).

In summary, arteriogenesis is potentially able to preserve the function of an occluded artery. The success of this remodeling process depends on close

coordination of a variety of mechanical and biochemical factors as discussed. Complete understanding of the molecular machinery for arteriogenesis is still elusive.

B. Arteriovenous (AV) Differentiation

During the early stage of embryogenesis, arteries and veins in the primary capillary plexus form via a process known as AV differentiation (Adams & Alitalo 2007, Jain 2003, Torres-Vazquez et al 2003). Growth and specification of arteries and veins continues throughout development and reflects distinct hemodynamic properties within the vascular architecture. Formerly it was believed that AV differentiation of ECs in the primary capillary plexus was due to the influence of hemodynamic forces (Dewey et al 1981). In 1998, Wang was the first to find that EphrinB2 and EphB4 are markers for arterial and venous ECs, respectively, in the primary capillary plexus (Wang et al 1998). This suggested that arterial and venous EC determination is at least partially governed by genetic factors prior to the establishment of circulation. Since then, numerous molecules and signaling pathways have been described that participate in AV differentiation (Table I.1 and Figure I.2). For example, arterial ECs express high levels of Notch1 and 4 (Villa et al 2001), Jagged1 and 2 (Villa et al 2001), Delta-like ligand 4 (Dll4) (Shutter et al 2000), Ephrin B2 (Wang et al 1998), Neuropilin 1 (Nrp1) (Mukouyama et al 2002) and Hey2 (Chi et al 2003); whereas venous ECs are characterized predominately by the expression of EphB4 (Wang et al 1998), Nrp-2 (Herzog et al 2001, Yuan et al 2002) and COUP-TFII (You et al 2005).

Arterial gene	Species
ALDHIA1	Human (Chi et al 2003)
Alk1	Mouse (Seki et al 2003)
CD44	Mouse (Fischer et al 2004), human (Chi et al 2003)
Connexin37	Mouse (Shin & Anderson 2005)
Connexin40	Mouse (Mukouyama et al 2002, van Kempen & Jongsma 1999)
CXCR4	Mouse (Ara et al 2005)
deltaC	Zebrafish (Smithers et al 2000)
Delta-like 4	Mouse (Shutter et al 2000)
Depp	Mouse (Shin & Anderson 2005)
EphrinB2	Zebrafish (Lawson et al 2001), chick (Moyon et al 2001, Othman-Hassan et al 2001), mouse (Adams et al 1999, Wang et al 1998)
EVA1	Human (Chi et al 2003)
Foxc	Mouse, Human (Seo et al 2006)
Gridlock/Hey2	Zebrafish(Zhong et al 2000), human (Chi et al 2003)
IGFBP-5P	Mouse (Shin & Anderson 2005)
ITM2A	Human (Chi et al 2003)
Jagged1	Mouse (Villa et al 2001)
Jagged2	Mouse (Villa et al 2001)
KRT7	Human (Chi et al 2003)
LIPG Lipase	Human (Chi et al 2003)
Neuropilin-1	chick (Herzog et al 2001, Moyon et al 2001), Mouse(Mukouyama et al 2002),
Notch1	Mouse (Villa et al 2001)
Notch4	Mouse(Villa et al 2001), human (Chi et al 2003)
Notch5	Zebrafish (Lawson et al 2001)
Sox	Zebrafish (Sakamoto et al 2007)
Tbx20	Zebrafish (Ahn et al 2000)
Unc5b	Mouse (Lu et al 2004)
Venous gene	Species
APJ	Mouse (Devic et al 1999)
COUP--TFII	Mouse (You et al 2005)
EphB4	Zebrafish (Lawson et al 2001), mouse (Adams et al 1999, Wang et al 1998), Xenopus (Helbling et al 2000), human (Chi et al 2003)
Flt4	Zebrafish (Thompson et al 1998), mouse (Kaipainen et al 1995)

GDF1	Human (Chi et al 2003)
Lefty1/2	Human (Chi et al 2003)
Myosin 1B	Human (Chi et al 2003)
Neuropilin-2	Chick (Herzog et al 2001), Mouse (Yuan et al 2002)
smoothened	Human (Chi et al 2003)
Tie2	Chick (Moyon et al 2001)

Table I.1: Molecules expressed preferentially in arterial and venous ECs

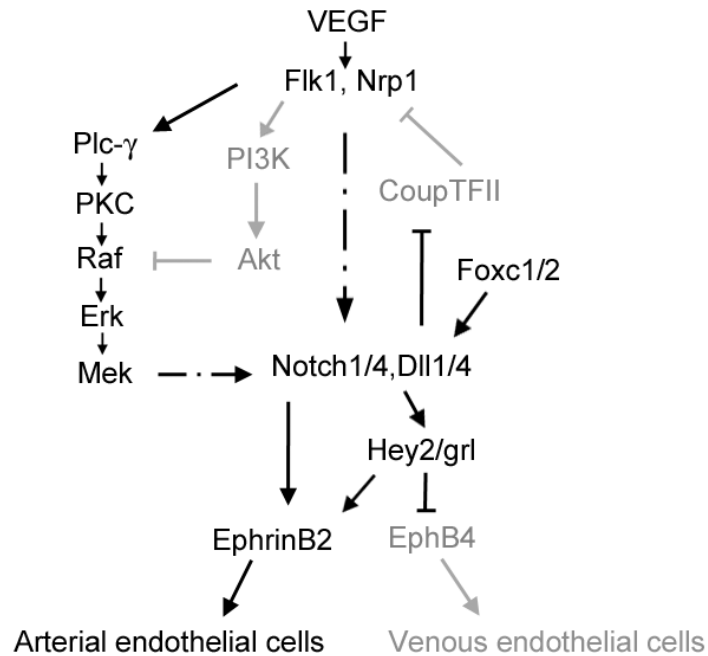


Figure I.2 Model of AV specification. VEGF interacts with the Flk-1-Nrp1 complex to activate downstream Plc-γ-Erk and Notch signaling pathways, thus inducing expression of arterial gene markers Hey2 and EphrinB2, while inhibiting expression of venous gene EphB4. Foxc proteins also activate the Notch pathway, leading to an arterial identity. Activated Notch can suppress COUP-TFII expression, thereby repressing a venous fate. Conversely, COUP-TFII can inhibit Nrp1 expression and thus attenuate VEGF signaling and downstream Notch activation. Moreover, the PI3K-AKT cascade represses Erk signaling. Therefore, a venous fate is promoted. Unconfirmed interactions are indicated by dashed arrows. The molecules and signaling pathways that favor an arterial fate are shown in black, while those that promote a venous fate are in grey.

Regulation of AV Specification

Ehprin/Eph

The first genes that were found to be expressed differentially in arterial and venous endothelium were Ephrin B2 and EphB4 (Adams et al 1999, Wang et al 1998), which are members of the Eph receptor tyrosine kinases (RTK) - Ephrin family. The ligand Ephrins are divided into 2 subclasses: the A-subclass (EphrinA1-A6), which anchored to the cell surface via a glycosylphosphatidylinositol (GPI); and the B-subclass (EphrinB1-B3), which has a transmembrane domain, followed by a short cytoplasmic region. Similarly, the Eph receptors are divided into A and B subclass (EphA1-A8 and EphB1-B6, respectively). The interactions of Ephrins and Ephs are prominent and are not class restricted (Klein 2004, Kullander & Klein 2002). Because Ephrins and Ephs are both membrane-bound, Ephrin-Eph signaling requires cell-cell contact. A unique feature of Ephrins is that they are capable of receptor-like active signaling. This results in bidirectional signal transduction, where conserved tyrosine residues in the approximately 85aa long cytoplasmic domain of Ephrin ligand are phosphorylated and recruit signaling effectors (Bruckner et al 1997, Holland et al 1996).

The distinct expression pattern of arterial EphrinB2 and venous EphB4 was first discovered in mice in the primary capillary plexus before initiation of circulation (Adams et al 1999, Gerety et al 1999, Wang et al 1998). Since then, it has been confirmed in chicks and zebrafish (Lawson et al 2001, Lawson et al 2002, Moyon

et al 2001, Othman-Hassan et al 2001, Zhong et al 2001). More importantly, the interaction of EphrinB2 with EphB4 is critical for proper vascular development. In knockout mice lacking EphrinB2, the primary capillary plexus forms, but its remodeling into a hierarchical, organized vasculature is arrested (Adams et al 1999, Wang et al 1998). Moreover, the dorsal aorta forms in these animals, while the cardinal vein remains a loose, nonfunctional network of ECs (Adams et al 1999). These two vessels initially are formed by vasculogenesis. Similar phenotypes are present in EphB4-deficient mice (Gerety et al 1999). Thus, EphrinB2 and EphB4 are not acquired for determining EC fate during vasculogenesis, but they are required to define and maintain the arterial and venous interface. Mice lacking the EphrinB2 cytoplasmic domain exhibit defects in vascular remodeling similar to those in mice that are completely deficient for EphrinB2 or EphB4. This suggests that their bidirectional signaling is also important for proper AV specification (Adams et al 2001).

Although EphrinB2 and EphB4 are markers of arteries and veins, studies in zebrafish and mice have demonstrated some other critically important upstream transcription factors that help determine arterial-venous EC fate, as discussed on the following pages.

Notch

The Notch signaling pathway is an evolutionarily conserved pathway that is involved in a variety of developmental processes. Notch family members and

Notch ligands are expressed throughout early vascular development and later are restricted to arteries (see Table I.1). Mice with targeted deletion of Notch molecules including Notch1 (Huppert et al 2000, Krebs et al 2000, Limbourg et al 2005), Notch4 (Carlson et al 2005, Krebs et al 2000, Uyttendaele et al 2001), Jagged1 (Xue et al 1999), Dll4 (Duarte et al 2004, Gale et al 2004, Krebs et al 2004), Rbpj (Krebs et al 2004), Hey1 and Hey2 (Fischer et al 2004, Kokubo et al 2005) display severely impaired vascular remodeling in the yolk sac and in the embryo proper, which indicates the essential roles of Notch molecules in vascular development. In particular, the role of Notch molecules in arterial specification has been extensively demonstrated in mice and zebrafish embryos, and in human ECs (Gridley 2007, Roca & Adams 2007, Rossant & Hirashima 2003, Rossant & Howard 2002, Shawber & Kitajewski 2004, Siekmann & Lawson 2007).

Although Notch4 knockout mice do not exhibit any apparent deficiencies in vessel formation, targeted deletion of Notch1 inhibits proper remodeling and embryos die *in utero*. Moreover, Notch1/Notch4 double-knockout mice display abnormal vascular development that is even more severe than that seen in Notch1 knockout mice (Krebs et al 2000), suggesting that Notch1 and Notch4 functions partially overlap. Dll4, a ligand of Notch1 and Notch4, is haploid-sufficient — loss of a single Dll4 allele results in reduced EphrinB2 expression and increased EphB4 expression, along with a failure in arterial differentiation (Duarte et al 2004). This defect is similar to that observed in Rbpj- and

Hey1/Hey2-knockout animals (Fischer et al 2004, Kokubo et al 2005, Krebs et al 2004). Most recently, Dll1 has been established as a requirement for maintaining arterial identity during fetal development and a mediator of postnatal arteriogenesis in mice with hindlimb ischemia (Limbourg et al 2007, Sorensen et al 2009). Collectively, these findings indicate Notch activity is important for promoting an arterial EC fate in mice.

Disruption of Notch signaling in zebrafish has many similar consequences as in mice. Mindbomb (mib) in zebrafish encodes a RING ubiquitin ligase that promotes ubiquitylation and internalization of Delta. The disruption of Notch signaling in mib mutant leads to reduced expression of arterial markers EphrinB2 and Notch5 and increases expression of venous marker Flt4 in the dorsal aorta. Similarly, overexpression of activated Notch5 in the posterior cardinal vein causes a decrease in Flt4 expression. The expression of Notch's downstream target gridlock (grl), a zebrafish orthologue of mammalian Hey2, is restricted to the dorsal aorta. Knockdown of grl represses Ephrin B2 expression and leads to an expansion of adjacent veins. Conversely, overexpression of grl suppresses venous growth with loss of Flt4 expression but does not affect dorsal aorta formation (Zhong et al 2001, Zhong et al 2000). Although grl is needed for arterial differentiation, its expression is not reduced in mib mutations or embryos injected with Su(H) (dominant negative suppressor of Hairless), suggesting grl might not be a direct target of Notch, making the role of grl in arterial specification in zebrafish obscure.

VEGF

VEGF is one of the most potent and ubiquitous vascular growth factors that affects many aspects of EC biology (Coultas et al 2005, Rossant & Hirashima 2003, Rossant & Howard 2002, Ruhrberg 2003). Consistent with VEGF's promotion of arterial EC differentiation in zebrafish, mice embryonic angioblasts treated with either VEGF₁₂₀ or VEGF₁₆₄ can induce an arterial EC expression pattern and undergo arterial specification (Mukouyama et al 2002). Similarly, overexpression of VEGF₁₆₄ in cardiomyocytes in transgenic mice results in an increase of EphrinB₂⁺ vessels (Visconti et al 2002). Interestingly, VEGF signaling in the regulation of arterial differentiation in mice is in an isoform-sensitive manner. Loss of VEGF₁₆₄ impairs retinal arterial endothelial outgrowth, whereas ablation of VEGF₁₆₄ and VEGF₁₈₈ perturbs renal arteriogenesis (Mattot et al 2002, Stalmans et al 2002).

Furthermore, recent work in zebrafish has shown Plc- γ /Erk under VEGF signaling favors an arterial EC specification. A molecular cascade (VEGF \rightarrow Plc- γ \rightarrow Pkc \rightarrow Raf \rightarrow Mek \rightarrow Erk) has been identified for promoting an arterial fate of ECs (Lawson et al 2003). In contrast, constitutive activation of PI3K/AKT induces venous EC fate, while repressing Erk activation (Hong et al 2006). Inactivation of AKT with flavone GS4898 reverses PI3K inhibitory effect on Plc- γ /Erk and thus promotes arterial EC specification (Hong et al 2006).

Sonic hedgehog (Shh)

The Hedgehog (Hh) family is composed of three ligands: sonic hedgehog, indian hedgehog and desert hedgehog. They all signal through the same receptors called PTCH1 and 2. The Hh signaling functions broadly in vascular development including coronary vascular development (Lavine et al 2008, Lavine et al 2006) and arterial specification (Lawson et al 2001). In zebrafish, loss of Shh activity either in the null mutant embryo sonic-you (syu) or in the embryo that treated with Shh signaling inhibitor cyclopamine results in loss of arterial identity and gain of venous marker expression (Lawson et al 2002). Conversely, overexpression of Shh leads to a switch from venous to arterial identity in posterior vein (Lawson et al 2002). In addition, regulation of arterial specification by Shh is critically associated with VEGF in the somite (Pola et al 2001). Microinjection of VEGF mRNA in Shh-deficient embryos can rescue arterial differentiation. However, injection of VEGF mRNA does not promote arterial differentiation in Notch-deficient embryos. Instead, activation of the Notch pathway in the absence of VEGF signaling can induce artery-specific gene expression (Lawson et al 2002). Together, these findings indicate that VEGF acts downstream of Shh and upstream of the Notch pathway to determine arterial EC fate.

Forkhead box c (Foxc)

The highly conserved Forkhead box (Fox) proteins are important in cardiovascular development (Kume et al 2001). Recent studies demonstrated that two Fox family members, Foxc1 and Foxc2, are essential for arterial

specification. *Foxc1/Foxc2*-null mice exhibit a fusion of the dorsal aorta with the posterior cardinal vein. In addition, these mutants lack induction of the expression of arterial markers including *Notch1*, *Notch4*, *Dll4*, *Jagged1*, *Hey2* and *EphrinB2*; whereas, venous markers such as *COUP-TFII* and *EphB4* are expressed normally, suggesting that mutant ECs fail to acquire an arterial fate (Seo et al 2006). Overexpression of *Foxc* genes in vitro consistently induces expression of arterial markers. The promoters of *Dll4* and *Hey2* are directly bound to and activated by *Foxc* proteins via a *Foxc*-binding site (Hayashi & Kume 2008, Seo et al 2006). Moreover, activation of *Dll4* and *Hey2* occurs to a greater extent when VEGF acts in combination with either *Foxc1* or *Foxc2*. Additionally, the transcriptional activity of *Foxc* proteins in *Dll4* and *Hey2* induction can be further modulated by VEGF-activated PI3K and ERK signaling pathways, making the regulation of AV specification via *Foxc* proteins more complicated. Collectively, *Foxc* transcriptional factors interact with VEGF and Notch signaling to regulate arterial gene expression.

Sry-related HMG box (Sox)

Transcription factors of the Sox family, such as *Sox7* and *Sox18*, are highly expressed in developing vasculature and have recently been identified to be involved in AV differentiation in zebrafish (Herpers et al 2008, Sakamoto et al 2007). Although disruption of either Sox protein does not cause any visible vascular defects, simultaneous blockage of *Sox7* and *Sox18* leads to severe AV malformation, characterized by vessel fusion and loss of circulation in the

posterior part of embryos (Herpers et al 2008). Further examination reveals that arterial specification of ECs is severely interrupted in double-knockdown embryos, which is often accompanied by failure of arteries to segregate from veins. Therefore, Sox7 and Sox18 are dispensable for vascular development individually, but synergistically they promote the specification of arterial ECs.

COUP- transcription factor 2 (COUP-TFII)

COUP-TFII, a member of the orphan nuclear receptor family, has been identified as the first regulator that positively mediates venous EC identity (You et al 2005). Targeted disruption of COUP-TFII results in embryonic lethality and displays a phenotype opposite of that observed in the loss of Notch activity: a partial loss of venous identity with reduced (but not completely abolished) EphB4 expression, accompanied by ectopic expression of arterial gene markers such as Notch1 and EphrinB2. Conditional lack of COUP-TFII in the endothelium causes veins to acquire an arterial identity. In contrast, ectopic expression of COUP-TFII in the endothelium results in AV malformation, which phenocopies the vascular deficiency observed in Nrp1- and Notch1-knockout mice. Moreover, overexpression of COUP-TFII represses the expression of Nrp1 and other arterial gene markers. It is now known that COUP-TFII suppresses Nrp1 expression by binding to the Nrp1 promoter. Thus, COUP-TFII is a critical factor that regulates the determination of venous EC fate.

Plasticity of AV Differentiation

Although it is now clear that the acquisition of arterial and venous identity is determined genetically, there is no doubt that epigenetic factors such as oxygen tension, blood flow and the vessel wall's microenvironment also can influence this process. Plasticity of ECs has been observed in the developing embryo by performing quail-chick grafting experiments (Moyon et al 2001, Othman-Hassan et al 2001). The quail arterial or venous vessel fragments, together with their vessel wall from various stages of embryonic development, were grafted into developing chick hosts. Quail ECs from arteries and veins were able to populate both host arteries and veins with equal efficiency, and the arterial marker expression changed with respect to the novel environment, regardless of its origin. However, this EC plasticity diminished progressively after E11 (Moyon et al 2001). Interestingly, removal of the vessel wall from the aorta, carotid artery and vena cava in E14 quail can fully restore the capacity of ECs to colonize to host arteries and veins, irrespective of their origin (Moyon et al 2001, Othman-Hassan et al 2001). These results suggest that specification of arterial and venous fate is, to some extent, reversible and the vessel wall may help stabilize the arterial and venous choice of ECs. How the vessel wall precisely affects endothelial AV determination is yet unknown.

EC plasticity with respect to AV differentiation also occurs during development according to blood flow. It is observed that initiation of arterial and venous marker expression in the primary capillary plexus occurs independently of flow (le Noble

et al 2004, Wang et al 1998). Shortly after the onset of circulation, the vitelline artery forms from the arterial capillary plexus in the posterior pole of the embryo by fusion of individual capillaries. But not all capillaries are involved in the formation of the artery. Some of them become disconnected, exhibit downregulation of arterial markers, and then serve to form the vitelline vein (le Noble et al 2004). Furthermore, ligation of the vitelline artery on one side of the yolk sac makes it become venularized over 24 hours, as judged by the direction of blood flow and the expression of arterial makers. Similarly, flow manipulation can switch veins to arteries (le Noble et al 2004). Therefore, hemodynamic forces also can alter EC AV identity.

Oxygen tension differs in arteries and veins, and this can be another potential instructive signal for AV differentiation. The influence of altered oxygen tension on AV differentiation has been observed in the developing mouse retinal vasculature (Claxton & Fruttiger 2005).

ECs in adults possess limited plasticity for AV differentiation, which has been demonstrated in vein graft adaptation in humans and aged rats (Kudo et al 2007). EphB4 expression was lost and intima-media thickening was observed, but arterial markers EphrinB2 and Dll4 were not strongly induced during vein graft adaptation to the arterial environments (Kudo et al 2007).

Taken together, recent studies have demonstrated that AV specification is genetically predetermined, and many molecules involved in the regulation of this process have been identified. In addition, environmental factors such as oxygen tension, hemodynamic forces and the microenvironment of vessel walls can further influence vascular identity in terms of forming and maintaining arteries and veins.

C. Endothelial Colony Forming Cells (ECFCs)

The concept that circulating bone-marrow-derived endothelial progenitor cells (EPCs) could contribute to neovascularization via postnatal vasculogenesis was first proposed in 1997 (Asahara et al 1997). Since then, EPCs have been studied widely and regarded as a mechanism for maintenance of vascular homeostasis. Defective regulation of EPCs leads to pathogenesis of various disorders. However, to date, no cell surface molecules that uniquely identify EPCs have been reported in human or other vertebrate species. This effort continues, as researchers seek to isolate and define EPCs based on in vitro adhesion and growth, and cell surface phenotype selection using fluorescent-labeled antibodies and flow cytometry (see Figure I.3) (reviewed in (Hirschi et al 2008, Yoder & Ingram 2009, Yoder et al 2007)).

In the “cell adhesion and growth” approach, low-density mononuclear cells from human peripheral blood are isolated and plated on fibronectin- and gelatin-coated dishes. After several days in culture, nonadherent cells are removed. The

adherent cells display the ability to ingest acetylated low-density lipoprotein (AcLDL) and bind to fluorescently labeled *Ulex europaeus agglutinin I* (UEA I) plant lectin and thus are deemed as EPCs. These putative EPCs are released from culture and undergo staining to confirm expression of endothelial markers such as von Willebrand factor (vWF), VE-Cadherin and KDR by flow cytometry. In additions, an inverse relationship between the circulating concentration of these EPCs and an increased risk for developing coronary artery disease in human subjects has been reported (Vasa et al 2001). However, others have shown that putative EPCs isolated in this method may also express, to varying degrees, CD45, CD11b, CD11c, CD14, CD68, eNOS, and E-selectin, and ingest India ink like macrophages do (Rehman et al 2003, Rohde et al 2007, Rohde et al 2006, Zhang et al 2006). Actually, the use of fibronectin- and gelatin-coated tissue culture plates has been recognized as a method to isolate human blood monocytes for differentiation into macrophages (Freundlich & Avdalovic 1983). Moreover, monocytes/macrophages are well known to express “endothelial-specific” proteins, particularly when cultured in conditions that promote endothelial growth. Therefore, the use of this method for isolating EPC is not reliable.

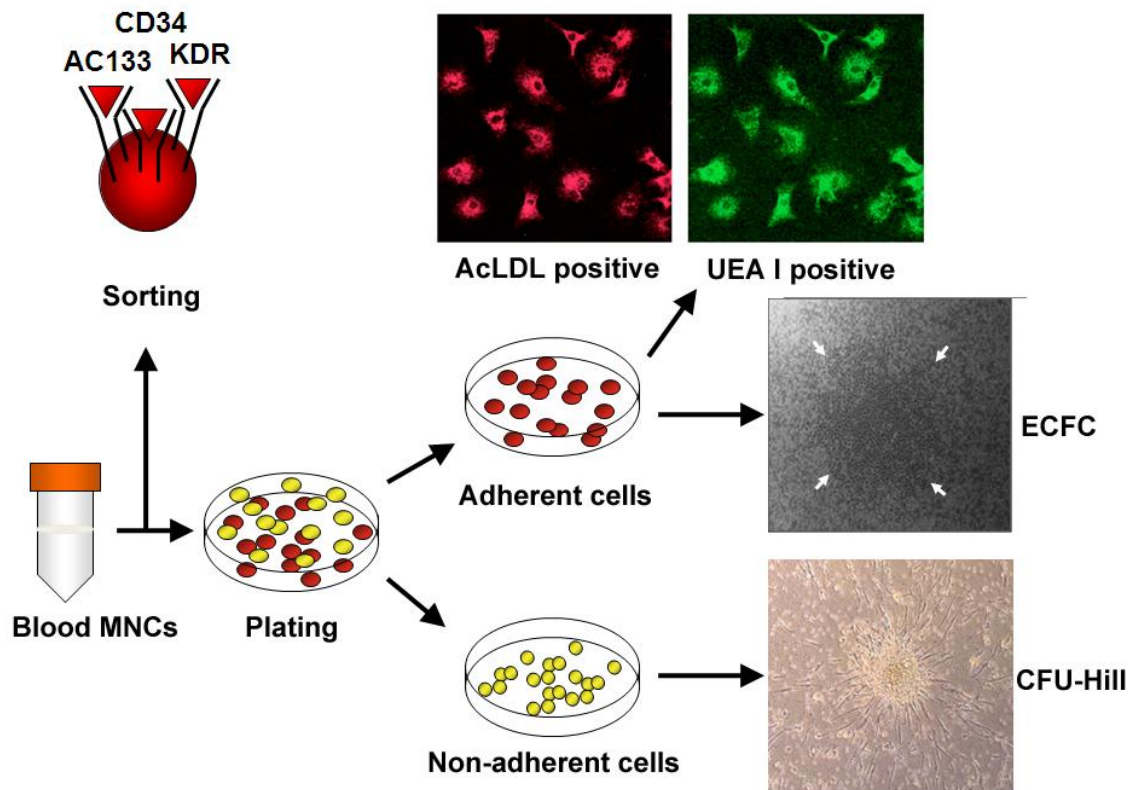


Figure I.3 Common methods of “EPC” culture. Culture of CFU-Hill cells includes a 5-day process where nonadherent peripheral blood MNCs plated on FN-coated dishes give rise to colonies. ECFCs are derived from adherent peripheral blood or cord blood MNCs cultured for 6 to 21 days on rat-tail collagen I coated dishes, and colonies display cobblestone morphology. When adherent peripheral blood MNCs cultured in FN-coated dishes, they typically do not display colony formation. These cells are able to ingest AcLDL and bind to UEA I lectin. The other method is to sort CD34+AC133+KDR+ cells directly from peripheral blood MNCs instead of plating them.

A second method utilizes fluorescent-labeled antibodies and flow cytometry analysis to enumerate putative EPC concentrations in human circulation. In the first EPC paper, Asahara (Asahara et al 1997) reasoned that putative circulating EPCs may express surface molecules shared by endothelial and hematopoietic stem/progenitor cells because these two lineages share a similar mesoderm origin during early embryogenesis. Thus, human circulating CD34+ cells were isolated. They reportedly adhered to fibronectin-coated plates with greater frequency than to type 1 collagen-coated plates and displayed a spindle-shaped morphology. The adherent putative EPCs expressed a variety of cell surface markers that normally were present in human umbilical vein endothelial cells (HUVECs). Further studies demonstrated that these putative EPCs (CD34+ or KDR+) home to areas of neovascularization when injected in vivo into immunodeficient mice with induced hindlimb ischemia. These studies collectively indicated that CD34+ or KDR+ circulating cells in human peripheral blood may be EPCs and can contribute to postnatal angiogenesis.

The number of endothelial cells circulating in the bloodstream in healthy subjects is very low, but can increase under certain conditions (Blann et al 2005). Peichev (Peichev et al 2000) attempted to define a panel of cell-surface antigens that may distinguish EPCs from circulating endothelial cells (CECs). Peichev utilized CD34, KDR, and AC133 as EPC markers. AC133, originally identified on neuroepithelial cells, is a 5-transmembrane domain cell-surface glycoprotein that is expressed in epithelial, hematopoietic, and various cancer stem cells (Mizrak

et al 2008). Peichev rationalized that the expression of CD34, KDR and AC133 should be present in EPCs and the expression of AC133 and CD34 would be downregulated in CECs, which is similar to gene expression observed in hematopoietic stem cells as they differentiate into more mature progenitors. Reportedly, a CD34+KDR+AC133+ population can be identified in adult peripheral blood, umbilical cord blood, and human fetal liver samples; but the concentration is very low. Subsequently, multiple combinations of CD34, AC133 and/or KDR have been used extensively to identify circulating EPCs in human subjects (reviewed by (Timmermans et al 2009)); and the concentration of circulating EPCs has been correlated with many human diseases (Bertolini et al 2006, Jujo et al 2008, Pompilio et al 2009). However, recent studies have been demonstrated that cells expressing CD34, AC133 and/or KDR are enriched for hematopoietic colony-forming cells and do not form endothelial cells in vitro and in vivo (Case et al 2007, Timmermans et al 2007). Thus, the expression of CD34, AC133 and KDR in human circulating cells fails to specifically identify a circulating EPC.

The third approach utilizes in vitro colony-forming assays to identify putative EPCs. Human peripheral blood mononuclear cells are placed on fibronectin-coated dishes for 48 hours; then the nonadherent cells are replated to quantify the emergence of the EPC colony forming units several days later. The putative EPCs (that give rise to progeny forming the colony) have been referred to as colony forming unit-Hill (CFU-Hill). The CFU-Hill assay has been used to

demonstrate a significant inverse correlation between the concentration of circulating CFU-Hill and Framingham cardiovascular risk scores in human subjects (Hill et al 2003). CFU-Hill has been recognized as being composed of an aggregate of round cells overlying adherent spindle-shaped cells expressing many proteins similar to the primary endothelial cells. However, these adherent spindle-shaped cells also express several myeloid progenitor cell markers and mature into macrophages that can readily ingest bacteria. In addition, these cells neither proliferate extensively to give rise to secondary colonies in vitro nor form blood vessels spontaneously when implanted in vivo in collagen gels (they do not display postnatal vasculogenic activity) (Yoder et al 2007). Furthermore, recent studies have demonstrated that CFU-Hill cells are mainly T cells and monocytes admixed with B cells and natural killer (NK) cells. The combination of purified T cells with monocytes is able to form CFU-Hill colonies (Rohde et al 2007, Rohde et al 2006). Thus, the CFU-Hill assay identifies hematopoietic cells instead of endothelial cells.

We and others have isolated ECFCs from human peripheral blood and cord blood (Au et al 2008a, Ingram et al 2004, Melero-Martin et al 2007). Human blood low-density mononuclear cells are placed on rat-tail collagen I coated dishes, and the nonadherent cells are removed. Several days later, ECFC colonies with the typical cobblestone morphology emerge in a medium that benefits endothelial cell growth. ECFCs express cell surface proteins (KDR, CD34, vWF, eNOS, VE-cadherin, and others) similar to those on primary ECs,

but ECFCs do not express hematopoietic cell markers such as CD45, AC133, CD11b and CD14 (Ingram et al 2004, Yoder et al 2007). They can proliferate at a clonal plating level and replate into secondary and tertiary ECFCs (Ingram et al 2004). They are able to incorporate AcLDL and form capillary-like structures in vitro (Ingram et al 2004). Most importantly, in sharp contrast to CFU-Hill and any other putative EPCs, ECFCs are capable of forming human blood vessels in vivo in immunodeficient mice and incorporate with murine vasculature to become part of murine systemic circulation (Yoder et al 2007). Thus, only ECFCs display all the property of EPCs.

In summary, the types of cells being considered as EPCs vary widely from study to study, causing controversies in this field. However, the strictest definition of an EPC is a circulating cell that 1) displays the ability to produce endothelial progeny that form endothelial tubes in vitro and 2) contribute to the functional endothelial lining of injured vascular structure via angiogenesis and/or vasculogenesis in vivo. Thus, to date, only human ECFCs fit the definition of true EPCs and display all the activities described on these pages.

CHAPTER II

A Hierarchy of Endothelial Colony Forming Cell (ECFC) Activity is Displayed by Bovine Corneal Endothelial Cells (BCECs)

Introduction

Corneal endothelial cells (ECs) are derived from neural crest precursors during embryonic development. These unique cells form a distinctive monolayer of hexagonally packed cells attached to their specialized basement membrane, Descemet's membrane, and form the most posterior portion of the cornea. Corneal ECs fail to proliferate in vivo in response to injury, disease, or aging and are arrested in the G1 phase of the cell cycle. However, corneal ECs possess replicative potential that can be revealed upon in vitro endothelial cell culture and/or application of a stress such as mechanical wounding or ethylene diamine tetraacetic acid (EDTA) treatment to disrupt cell-cell interactions within the endothelial monolayer on the cornea. A number of laboratories have also reported successful growth of untransformed corneal ECs in vitro. Using in vitro culture approaches, recent studies demonstrate that the proliferation of corneal ECs varies with the age of the donor (cells derived from younger donors divide more than those from older donors) and varies from the central cornea (low proliferative potential) to peripheral cornea (high proliferative potential). To date, it remains unclear if each corneal EC displays proliferative potential in vitro or if the replicative ability resides in only a subset of the cells.

We have reported on development of methods to examine clonal proliferative behavior of circulating and vascular endothelial cells and have identified a hierarchy of endothelial colony forming cell (ECFC) activity ranging from high proliferative potential ECFC (HPP-ECFC) to non-dividing mature ECs (Ingram et al 2005b, Ingram et al 2004). Human umbilical cord blood is enriched in circulating ECFCs and the distribution of ECFCs is skewed to a high percentage of HPP-ECFCs compared to human adult peripheral blood samples (Ingram et al 2004). Both cord blood and adult peripheral blood ECFCs display phenotypical and functional properties which are also present in ECs derived from human blood vessels (Ingram et al 2005b). Furthermore circulating or resident ECFCs form human blood vessels de novo when subcutaneously implanted into immunodeficient mice and these vessels participate in carrying blood as a part of the host murine systemic circulation (Au et al 2008a, Schechner et al 2000, Yoder et al 2007). These current studies suggest that ECFCs represent stem/progenitor cells for the endothelial lineage.

We questioned whether corneal ECs, although derived from neural crest rather than mesoderm and differing in anatomic position, exposure to blood flow and blood constituents, and displaying unique physiological functions, displayed clonal ECFC potential. We report that BCECs display a complete hierarchy of ECFCs that is similar to the distribution of ECFC activity in ECs isolated from bovine aorta, coronary artery, and pulmonary artery. HPP-ECFCs in corneal endothelium can be replated into at least secondary colonies and retain high

levels of telomerase activity similar to HPP-ECFCs derived from resident endothelium in blood vessels. These data suggest that the fundamental paradigm for endothelial cell lineage development and maintenance may be similar in corneal ECs as with other vascular ECs, but the mechanisms for EC repair and regeneration are regulated by dominant tissue specific requirements and niches.

Materials and Methods

Isolation of bovine peripheral blood mononuclear cells (MNCs)

Blood (50-100ml) was collected from a local slaughterhouse and diluted one to one with Dulbecco's Phosphate-Buffered Saline (DPBS) (1X) without Ca or Mg (Invitrogen, Grand Island, NY)/1% bovine serum albumin (BSA)/ethylenediamine tetraacetic acid (EDTA) solution and layered (2:1 ratio) onto Histopaque1119 (Sigma-Aldrich, St Louis, MO). Cells were centrifuged for 30 minutes at 1800 rpm at room temperature (Beckman Coulter, Fullerton, CA). Low density mononuclear cells (MNCs) were isolated as the floating monolayer and washed three times with DPBS. Finally, MNCs were resuspended in DPBS with 2% fetal bovine serum (FBS) (Hyclone, Logan, UT) for direct analysis by fluorescence activated cell sorting (FACS).

Culture of bovine vessel derived endothelial cells (ECs)

Bovine aortic endothelial cells (BAECs), bovine coronary arterial endothelial cells (BCAECs) and bovine pulmonary arterial endothelial cells (BPAECs) were purchased from Lonza (Lonza, Walkersville, MD). These bovine vascular endothelial cells were plated onto type I rat tail collagen (50 µg/ml) (BD Biosciences, Bedford MA) pre-coated tissue culture flasks and cultured in endothelial cell growth medium EGM-MV (Lonza) supplemented with 1.5% antibiotic-antimycotic (Invitrogen) at 37°C, 5% CO₂ in a humidified incubator.

Isolation and culture of bovine corneal ECs

The isolation of bovine corneal endothelial cells (BCECs) was performed as previously described (Bonanno and Giasson, 1992). These corneal ECs were plated in tissue culture flasks filled with DMEM supplemented with 5% FBS and 1.5% antibiotic-antimycotic. Corneal ECs were grown to a confluent monolayer and the cells displayed a typical hexagonal appearance. These ECs were released from the culture dish by using TryPLE (Gibco, Grand Island, NY) and re-plated onto 75 cm² tissue culture flasks for further passage in the supplemented DMEM.

Immunophenotyping of endothelial cells

Early passage (2-4) bovine vascular ECs and corneal ECs as well as bovine blood MNCs were stained with a mouse monoclonal antibody against bovine CD45 conjugated to fluorescein isothiocyanate (FITC) (SEROTEC, UK), FITC conjugated *Lycopersicon Esculentum* (Tomato) lectin (LEL) and *Griffonia simplicifolia* I lectin (GSL I) (Vector Laboratories, Burlingame, CA) or mouse isotype control antibody for 30 minutes at 4°C, washed three times, and analyzed for cell surface expression using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA) as previously described (Huang et al 2007).

Ingestion of 488-conjugated acetylated-low density lipoprotein (488-AcLDL)

Early passage (2-4) bovine vessel wall derived ECs and corneal ECs were incubated with 10 µg/mL of 488-AcLDL (Invitrogen) in the media for 8 hours at

37°C. Cells were washed three times, co-stained with 1.5µg/mL of the nuclear stain, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma), and examined by inspection through an inverted fluorescence microscope (Zeiss, Thornwood, NY) at 40 x magnification.

Tube formation assay

Capillary tube formation by the various ECs was performed as previously described using Matrigel coated dishes (Ingram et al 2004). Early passage (2-4) bovine vascular ECs and corneal ECs were seeded onto 96-well tissue culture plates coated with 30µl Matrigel (BD Biosciences) at a cell density of 10,000-30,000 cells per well. Cells were observed every two hours by an inverted microscope for the formation of capillary-like structure.

RT-PCR

Total cellular RNA was extracted with TRIzol (Invitrogen) in a single-step method as described by the manufacturer. RT reactions were performed using a SuperScript™ First-Strand Synthesis system (Invitrogen). PCR was conducted using Go Tap Flexi DNA Polymerase (Promega, Madison, WI) according to the manufacturer's instructions. The primer sequences were shown in Table II.1. The PCR cycle was 94°C, 5 min; 94°C 30s, 53 or 57 or 59°C (depending on the different primers) 30s, 72°C 45s, and 32 cycles with a final 72°C 7 min cycle.

PCR products were added to wells in a 2% agarose/ethidium bromide gel, exposed to electrophoresis current, and migrating bands were photographed under UV light.

Gene	Forward	Reverse	Tm	Product size
S100B	GGTGACAAGCACAAAGCTGAA	CAGTGGTAATCATGGCAACG	55	184
Enolase 2	GGACTTGGATGGGACTGAGA	GCGTCCTTGCCATACTTGTC	57	327
N-Cadherin	AGCAACTGCAATGGGAAAAG	GATGGGAGGGATAACCCAGT	55	461
VE-Cadherin	GAGTGTGGACCCCAAGAAGA	GCTGGTACACGACAGAAGCA	57	359
eNOS	TGAGCAGCTGCTGAGCCAGG	CAGCTCGCTCTCTCGGAGGT	57	209
PECAM I	ATGTGCTGCTTCACAACGTC	TGAATTCCAGCGTCACAAAA	53	345
Vimentin	CTTCGCCAACTACATCGACA	GGATTCCACTTTACGCTCCA	55	340
Enolase 1	GCAGGAGAAGATCGACAAGC	GTAAACCTCTGCTCCGATGC	57	310
ZO 1	GAATCCGATGTGGGTGATTC	GCAGGTTTCTCTTGAGCTG	57	310
Factor VIII	ACTGCCTCATCCCCTTACG	GGGGTCTAGAGCATTACCA	57	327
GAPDH	GGTGAAGGTCGGAGTGAACG	GGGTCATTGATGGCGACGA	59	117
LDLR	ACAACCCCGTGTACCAGAAG	AGGGTCAGGGGAGAAAGTGT	57	195
CD36	AACCACTTTCATCAGACCCG	ACGTGTCATCCTCAGTTCCA	57	475

Table II.1: Primers used for conventional RT-PCR

Single cell clonogenic assay

Early passage (2-4) bovine vascular ECs and corneal ECs, were selected by size and cell complexity using a FACS Vantage Sorter (Becton Dickinson) and deposited as one cell per well into 96 well plates pre-coated with type I rat tail collagen in 200 µl of media as previously described (Huang et al 2007). Cells were cultured at 37°C, 5% CO₂ in a humidified incubator. Media was changed every five days. After 14 days of culture, the cells were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) in DPBS for 30 minutes at room temperature, then washed twice and stained with 1.5 µg/ml DAPI, and examined for determination of EC number. Those wells containing two or more cells were identified as positive for proliferation under a fluorescent microscope at 10x magnification. Culture wells containing fewer than 50 cells were counted by direct visual inspection with a fluorescent microscope at 10x magnification. For those wells with more than 50 cells, colonies were imaged and cell number quantified using an Image J 1.36v program (Wayne Rasband, NIH).

Sphere forming assay

Early passage (1-2) bovine corneal ECs were released from adhering to the culture dish using TryPLE and then suspended into single cells in the culture medium. Cells were seeded at a density of 50 cells/µl on plastic dishes as hanging drops (10 µl each) to allow spheroid formation by cell aggregation. Cells then were cultured at 37°C, 5% CO₂ in a humidified incubator 3 days. Under this

condition, the suspended cells contributed to formation of a single spheroid per drop of defined size (diameter > 100µm).

Telomerase assay

Telomerase activity was measured by the telomeric amplification protocol (TRAP) as previously described (Kim et al 1994) using the TRAPeze telomerase detection kit (Chemicon, Temecula, CA). Cells lysate from 1000 cells were used in each assay. Hela cell extract served as a positive control and lysis buffer only as negative control. The PCR products were exposed to an electrophoretic current on a 12.5% non-denaturing polyacrylamide gel and visualized by SYBR gold staining (Molecular Probe, Eugene, OR).

Statistical Analysis

Results are expressed as mean±SEM for the study variables. Data were compared with ANOVA test and significant differences were set at the $P < 0.05$ level. All analyses were performed using GraphPad InStat software (GraphPad Software Inc, La Jolla, CA)

Results

Characterization of BCECs compared to bovine vascular endothelial cells

BCECs have been successively isolated and expanded in vitro. We isolated and cultured BCECs from 18 and 24 month old mixed breed steers and identified the samples as BCEC 18 and BCEC 24, respectively. These primary cultured BCECs displayed a typical hexagonal morphology (similar to the endothelial morphology in the intact cornea by direct visualization) at confluence. In comparison, bovine ECs from aorta, coronary artery, and pulmonary artery exhibited more morphological heterogeneity with more spindle-shaped and large oval cells (Figure II.1).

To determine whether ECs displayed any common hematopoietic antigens, we examined BCECs as well as bovine vascular ECs for expression of the leukocyte common antigen CD45 (known to be expressed on all nucleated blood cells). None of the endothelial cells expressed CD45 while the peripheral blood low density MNC (comprised of leukocytes) highly expressed CD45 (Figure II.2A). BCECs and bovine vessel wall derived ECs were able to bind LEL while peripheral blood MNCs did not display this activity (Figure II.2A). Additionally, BCECs, vascular ECs, and MNCs were all able to bind GSL I (Figure II.2A). Actually, BCECs and bovine vascular ECs had the same lectin binding pattern for all 30 lectins tested (data not shown). Thus BCECs share some cell surface molecule expression with bovine vessel wall derived ECs but there is no

evidence that any of the ECs express the most common hematopoietic antigen CD45.

Ingestion of AcLDL is a feature displayed by many ECs and not surprisingly, all bovine vessel wall derived ECs readily ingested the lipoprotein complex (Figure II.2B). In contrast, BCECs were unable to ingest AcLDL (Figure II.2B). The LDL receptor (LDLR) and scavenger receptors, such as CD36, mediate native and modified LDL-ingestion. We further examined LDLR and CD36 expression in bovine vascular and corneal endothelial cells after exposing the endothelial cells to AcLDL. LDLR transcripts were detectable in both types of ECs while CD36 transcripts were only present in vascular ECs after 8h of exposure to AcLDL (Figure II.2C). Capillary-like tube formation is another feature displayed essentially by all vascular ECs when examined in vitro. Surprisingly, BCECs, similar to all of the bovine vascular ECs, readily formed capillary-like structures when plated on Matrigel-coated dishes (Figure II.2D). Therefore, while BCECs always exist as a flattened monolayer lining the most posterior aspect of the cornea in vivo, removal of the cells and replating in vitro permits some functional properties of vascular ECs to emerge from the corneal ECs.

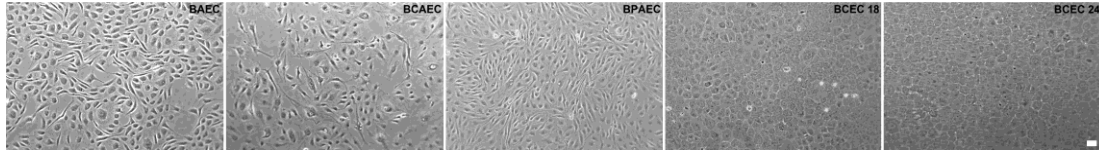


Figure II.1 The morphology of cultured bovine vessel wall derived ECs and bovine corneal ECs. Bovine vascular ECs derived from aorta (BAECs), coronary artery (BCAECs) and pulmonary artery (BPAECs) display spindle shape morphology and BCECs present a hexagonal monolayer. (10x magnification, scale bar represents 100 μ m)

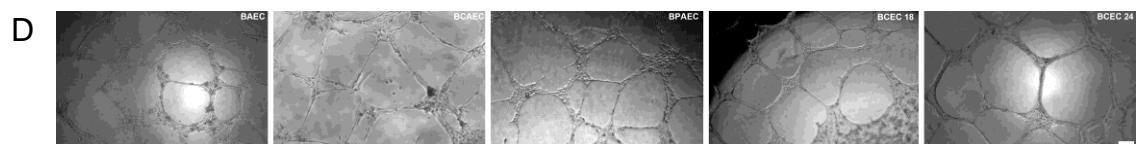
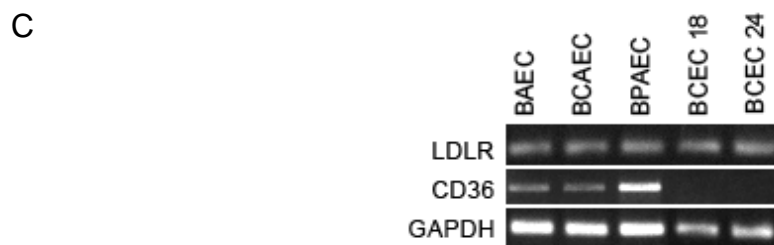
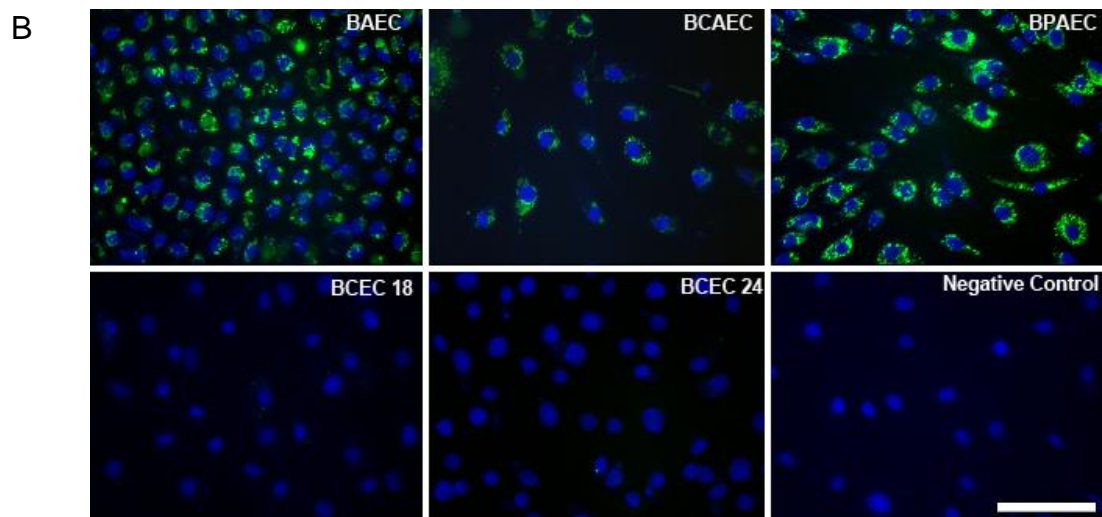
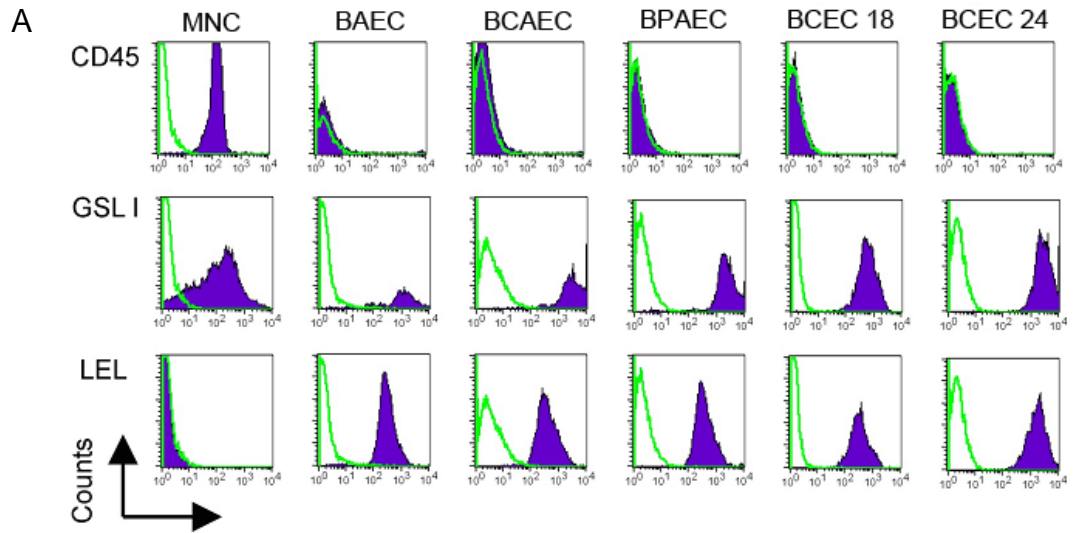


Figure II.2 Phenotypic and functional characterization of bovine vessel wall derived ECs and bovine corneal ECs. (A) Immunophenotyping of bovine peripheral blood MNCs and the monolayers derived from BAECs, BCAECs, BPAECs and BCECs by fluorescence cytometry. BAECs, BCAECs, BPAECs and BCECs can bind to the lectins GSL I and LEL but do not express common leukocyte antigen CD45. Negative controls are overlayed in green on each histogram. (B) Incorporation of 488-AcLDL in bovine vessel wall derived ECs and corneal ECs. BAECs, BCAECs and BPAECs are able to ingest 488-AcLDL (green) but not BCECs. The cell nuclei are stained with DAPI (blue). (40x magnification, scale bar represents 100µm) (C) LDLR and CD36 expression in bovine vascular and corneal ECs after exposed to AcLDL using RT-PCR. LDLR is expressed in all types of ECs while CD36 is only detectable in bovine vascular ECs after ingesting AcLDL. (D) Formation of capillary-like structures when bovine vascular ECs and BCECs are plated in Matrigel. (40x magnification, scale bar represents 100µm) Three independent experiments show similar results.

Gene expression of BCECs compared to bovine vascular endothelial cells

Although corneal ECs and vascular ECs possess differences in developmental origin, anatomic location, and physiological function, they have showed some similarities in phenotype and function in the above studies. To further compare and contrast these sources of ECs, we interrogated all of the EC lines for the expression of certain mRNA transcripts that are published as commonly expressed in all vascular endothelial cells (Figure II.3) (Albelda et al 1991, Huang et al 2007, Ohashi et al 2007). Remarkably, BCECs did not express vascular endothelial-cadherin (VE-Cadherin), nitric oxide synthase 3 (endothelial nitric oxide synthase; eNOS) or platelet/endothelial cell adhesion molecule 1 (PECAM1) which are expressed in bovine vascular ECs derived from aorta, coronary artery, and pulmonary artery. While there has been no single specific genetic marker for corneal ECs yet identified, we have scanned the published literature and identified numerous genes, including S100B, enolase2, N-cadherin, vimentin, ZO-1 and factor VIII, which are reportedly expressed preferentially in corneal ECs. S100B mRNA was present in BCECs and BPAECs, but absent in BAECs and BCAECs. Neuron specific enolase 2 mRNA was present in BCEC 18 and BCAECs and in three experiments, detectable in BCEC 24 but at the low level of detection. N-Cadherin mRNA was present in all ECs except BAECs. Furthermore, vimentin, enolase1, ZO1 and factor VIII mRNA were detectable in all ECs. Thus, BCECs displayed a gene expression profile that differed somewhat from bovine aortic, coronary artery, and pulmonary artery ECs, however, no one single gene product was BCEC specific. Whether these

differences are related to the known influence of in vitro culture on modulating gene expression and cell function in all the endothelial cells tested or truly represent tissue specific differences in gene expression will require further search for specific markers for these different endothelial populations that permits prospective isolation from each tissue and gene expression interrogation.

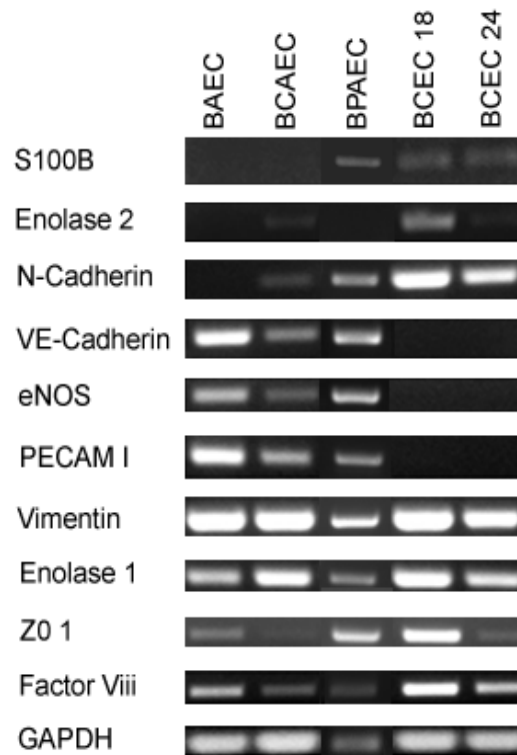


Figure II.3 RT-PCR analysis of gene expression in bovine vessel wall derived ECs and bovine corneal ECs. PECAM1, VE-Cadherin and eNOS are specifically expressed in bovine vascular ECs but not in corneal ECs. S100B transcripts are detectable in BCECs but not in vessel wall derived ECs. Enolase2 are also can be detected in BCECs with variable expression level. The other genes related to corneal ECs can also be detected in vascular ECs. Three independent experiments show similar results.

Hierarchical organization of the proliferative potential in BCECs and bovine vascular endothelial cells

We have recently developed a single cell clonogenic assay to quantitate the proliferative capacity of individual ECs. Using this method, we have defined a hierarchy of ECFCs present in both circulating and vessel wall derived ECs in human subjects and other vertebrates. Here we tested whether bovine vessel-derived ECs and corneal ECs possessed a similar hierarchy of cells with varying levels of proliferative potential. It was apparent that vascular ECs and corneal ECs displayed diverse proliferative ability at a clonal level. Some ECs did not divide and some divided and gave rise to various sized colonies of endothelium (Figure II.4). Significantly more single BAECs executed at least one cell division during 14 days of culture compared to BCECs (BAECs vs. BCEC 18 vs. BCEC 24 was $65.67 \pm 2.52\%$ vs. $32.63 \pm 1.22\%$ vs. $41.40 \pm 5.24\%$). However, there was no significant difference in the frequency of dividing cells among BAECs, BCAECs ($48.00 \pm 4.67\%$) and BPAECs ($46.93 \pm 7.46\%$) or among BCAECs, BPAECs and BCECs (Figure II.4A). Surprisingly, $39.82 \pm 2.71\%$ of the individual BCEC 18 and $43.25 \pm 3.49\%$ of the individual BCEC 24 cells that divided gave rise to well circumscribed colonies containing more than 10,000 progeny in the 2 weeks' assay. This frequency was significantly higher than that measured for individually plated BAECs ($33.67 \pm 4.22\%$). Differences in the ECFC distribution were observed in BCEC 18 cells versus BCEC 24 cells (at the same passage number), suggesting that some variability may be observed between donors with respect to this kind of distribution analysis.

We recently defined HPP-ECFCs as cells that can yield macroscopic colonies (at least more than 2000 cells) and can also form secondary HPP-ECFC colonies upon replating. To examine whether any BCECs or bovine vascular ECs possess HPP-ECFC activity, the progeny of primary ECFC colonies containing more than 10,000 cells were trypsinized and replated into 96-well tissue culture plates at one cell per well. After another 14 days of culture, the individually replated cells generated all sizes of colonies including some colonies containing more than 10,000 cells (data not shown). Thus a complete hierarchy of ECFCs was identified in BCECs and bovine vascular ECs, and these ECFC-derived progeny were comprised of non-dividing mature ECs, endothelial clusters (2-50 cells/colony), low proliferative potential-ECFCs (LPP-ECFCs, 51-2000 cells/colony) and HPP-ECFCs (2001 or greater cells/colony).

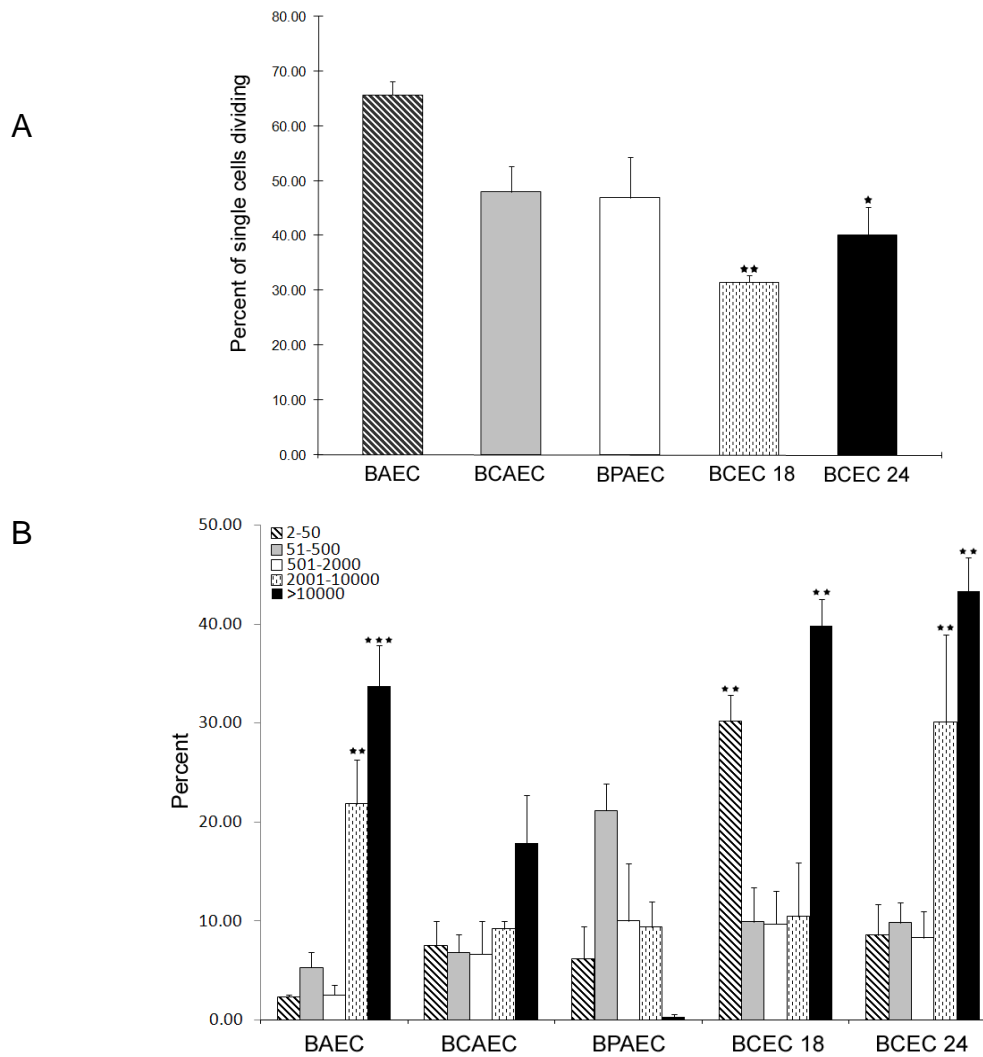


Figure II.4 Quantitation of the clonogenic and proliferative potential of single endothelial cells derived from bovine vascular endothelium and bovine corneal endothelium. (A) The percentage of single BAECs, BCAECs, BPAECs and BCECs dividing at least once after 14 days culture. There are significantly fewer BCECs undergoing division comparing to bovine vessel wall derived ECs. (B) The distribution of different size of colonies derived from single ECs in an individual well after 14 days of culture. There is dramatically higher percent of HPP-ECFCs than LPP-ECFCs and endothelial clusters in BAECs and BCECs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by parametric ANOVA. (n=3)

High levels of telomerase activity in BCECs and bovine vascular endothelial cells

We have previously reported that human cord blood HPP-ECFC possesses high levels of telomerase activity. Remarkably, the progeny of HPP-ECFC isolated from BCECs displayed telomerase activity similar to that of the progeny of HPP-ECFC derived from BAECs, BCAECs and BPAECs (Figure II.5). Thus, HPP-ECFCs in both BCECs and bovine vascular ECs express quantifiable levels of telomerase activity suggesting a potential mechanism through which proliferative potential is retained within the subset of endothelial cells that possess proliferative potential.

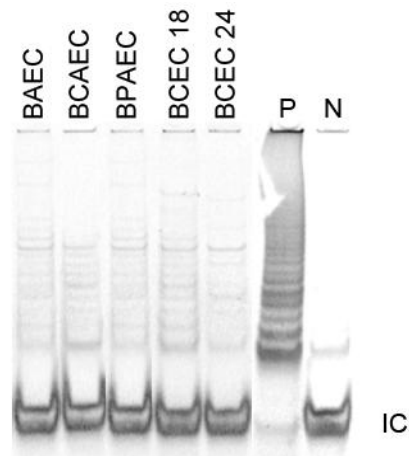


Figure II.5 Telomerase activity of HPP-ECFCs derived from bovine vessel wall derived ECs and BCECs. P indicates telomerase activity in Hela cells, which acts as a positive control, N indicates a negative control and IC indicates internal control. Three independent experiments show similar results.

Sphere forming ability displayed by BCECs

Human and rabbit corneal ECs have been reported to form sphere colonies in vitro (Mimura et al 2005, Yokoo et al 2005). Sphere formation has often been used as a surrogate assay to reflect the presence and frequency of stem and/or progenitor cells for the lineage under investigation (Ramirez-Castillejo et al 2006, Reynolds & Weiss 1992, Tropepe et al 2000, Xu et al 2009). Herein, we described that low passage cultured BCECs were able to form in vitro spheres (Figure II.6A) with an excellent efficiency of 53 ± 10 spheres per 100,000 cells after 3 days of culture. To interrogate the spheres for evidence of the clonal proliferative potential of the endothelial cells, 3 day old primary spheres were dissociated and cells were plated into a single cell clonogenic assay. Among 3000 sphere-derived single BCECs plated, only $9.58 \pm 6.09\%$ survived at the single cell level. However, $90.43 \pm 6.56\%$ of the surviving cells demonstrated the ability to divide at least once and form ECFC-derived colonies of varying sizes. We noted that the complete hierarchy of ECFCs was identified in sphere-derived BCECs and the distribution of proliferative potential was similar to that in primary cultured BCECs (Figure II.4B). Thus, these corneal endothelial spheroids are comprised of non-dividing mature ECs, endothelial clusters, LPP-ECFCs, and HPP-ECFCs (Figure II.6B). Therefore, while the majority BCECs that formed spheres in vitro failed to survive at a single cell level, those surviving cells retained a similar distribution of proliferative potential as the BCECs used to establish the in vitro sphere.

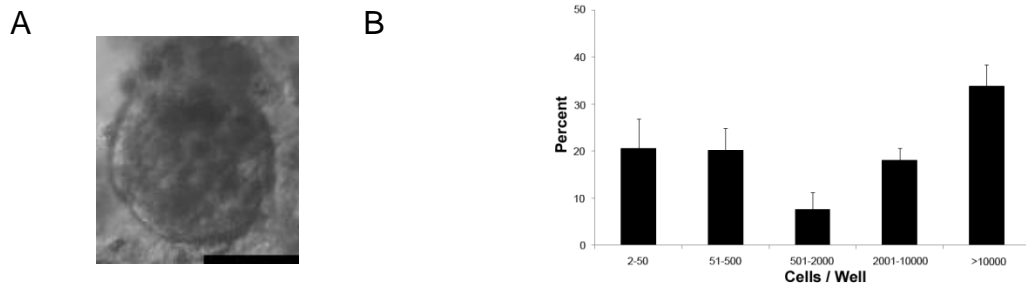


Figure II.6 The formation of sphere colony by bovine corneal endothelial cells. (A) Representative photograph of sphere derived from plated BCECs. (Scale bar represents 100µm) (B) The distribution of different size of colonies derived from single BCECs dissociated from corneal endothelial sphere colonies in an individual well after 14 days of culture. The complete hierarchy of ECFC is present in BCECs residing in the spheres.

Discussion

It is well known that corneal ECs are apparently restrained from proliferating in vivo, but they retain proliferative potential and can be expanded in vitro. The present study is the first to demonstrate that not all corneal ECs display the same ability to proliferate at a clonal level. In fact, we define a hierarchy of ECFCs in bovine corneal ECs based on their proliferative potential using a single-cell clonogenic assay. We report that the distribution of ECFCs derived from single BCECs is quite similar to bovine vascular endothelium from aorta, coronary artery, and pulmonary artery and is consistent with circulating and resident vascular endothelial cells in adult human subjects.

The proliferative activity of corneal endothelium in vitro has been observed under a variety of in vitro conditions. EDTA can release endothelial cells from contact inhibition and promote proliferation within the endothelial monolayer (Joyce & Zhu 2004, Senoo et al 2000). Cytokines such as epidermal growth factor (EGF) and nerve growth factor (NGF) can also induce corneal ECs proliferation in vitro (Joyce & Zhu 2004). Furthermore, the overexpression of oncogene proteins (SV40 large T antigen or E6/E7) or the transcription factor E2F2 in corneal ECs can induce proliferation (Joyce 2003, McAlister et al 2005). These observations indicate that at least some corneal ECs inherently possess high proliferative capacity. Most recently, Mimura and colleagues (Mimura & Joyce 2006) have reported that peripheral corneal ECs display more proliferative capacity than those more centrally located. Moreover, Mimura and Yamagami (Mimura et al

2005, Yamagami et al 2007) demonstrated that there are a greater number of corneal EC precursor cells in the periphery than in the center by performing a sphere-forming assay, and concluding that the higher frequency of sphere-forming ability in the periphery must be related to the retention of precursor cells. However, these important studies did not use a single cell clonogenic assay, which can quantitatively and stringently interrogate the proliferative potential of individual ECs, to test whether corneal ECs harbored different populations that could be distinguished by their clonogenic potential.

Since we previously identified the entire hierarchy of ECFCs in circulating and vessel-derived ECs in human, pig and rat, it's not surprising that this hierarchy of proliferation is also present in bovine vascular ECs. However, it is of interest that corneal ECs can also be discriminated by similar proliferative and clonogenic properties. In the present study, we have provided evidence that corneal endothelial HPP-ECFC give rise to all subsequent stages of ECFC development. Thus, corneal endothelium possesses the complete hierarchy of ECFCs, and HPP-ECFCs display properties of a corneal endothelial progenitor cell as they have the most proliferative capacity and can be cultured in vitro in the absence of stroma cells.

The existence of stem cells for the corneal endothelium has been reported recently. By using telomerase activity assay and BrdU (bromodeoxyridine) incorporation assay, Whikehart et al (Whikehart et al 2005) argued that the

putative stem cells for corneal endothelium are located in a niche at the posterior limbus between the peripheral endothelium and the trabecular meshwork (TM). More recently, McGowan et al (McGowan et al 2007) provided further evidence for the presence of such a population in this area by demonstrating the expression of stem cell markers (nestin, Oct-3/4 and Wnt-1) in the unwounded cornea. They observed that the differentiation markers (Pax-6, Sox-2) were induced in this area and extended to peripheral corneal endothelium after wounding, which suggested that these putative stem cells respond to corneal damage and initiate endothelial repair. However, Mimura and Yokoo recently demonstrated that they were able to isolate corneal endothelial precursor cells from corneal endothelium with a sphere-forming assay and that the corneal endothelium harbors precursor cells which exhibit high proliferative capacity, form colonies in a sphere-forming assay, and have self-renewal ability to give rise to the secondary colonies (Mimura et al 2005, Yokoo et al 2005). Additionally, the sphere-forming ability of the peripheral corneal ECs was reported to be greater than centrally located ECs (Mimura et al 2005, Yamagami et al 2007). Moreover, the progeny of isolated spheres display hexagonal morphology and transport activity.

Although a sphere-forming assay has been extensively utilized to quantitatively measure stem cell frequency in many fields including neural stem cells and cancer stem cell (Ramirez-Castillejo et al 2006, Reynolds & Weiss 1992, Tropepe et al 2000, Xu et al 2009), recent studies have indicated that not all cells

capable of forming a sphere meet the criteria for a stem cell (Singec et al 2006). Moreover, the in vitro conditions to form spheres often permits the highly motile spheres to merged together, which argues against these spheroid structures arising from a single stem cell (Singec et al 2006). Therefore, use of a sphere-forming assay may not permit accurate determination of which individual cells display proliferative potential. In our current study, we utilized a single cell clonogenic assay to provide direct evidence that corneal EC precursors exist in corneal endothelium. HPP-ECFCs display the property of corneal endothelial progenitor cells as they have the most clonal proliferative capacity, can be replated to form secondary HPP-ECFC, and give rise to all other ECFC colonies and/or mature non-dividing endothelium. Whether the peripheral corneal endothelium harbors more HPP-ECFCs than the central endothelium needs to be further examined and is currently being investigated in our lab.

In this study, we did not directly isolate sphere colonies from bovine corneal endothelium. Instead, we tried to generate corneal endothelial spheroids from plated BCECs from low passage cultures (passage 1 or 2). We noticed that without addition of fetal calf serum to the culture medium, isolated cells only aggregated but did not form spheres. Furthermore, when plated in the floating culture method as previously described (Mimura et al 2005, Yamagami et al 2007, Yokoo et al 2005), BCEC frequently became free of the sphere and reattached to the culture substrate. Thus, we had to use a hanging drop method of sphere formation that was modified from previous publications (Del Duca et al 2004,

Timmins & Nielsen 2007). Under these culture conditions, BCECs were able to form numerous spheres when 500 cells were deposited per drop of medium. This number is much lower than reported for a human corneal endothelial cell sphere-forming assay, where 257 ± 83 spheres were generated from 50,000 cells after 10 days of culture (Yokoo et al 2005). This might be due to obvious differences in: 1) culture conditions, 2) species, 3) use of freshly isolated corneal ECs versus plated corneal ECs. It is of interest that BCECs residing in the spheres displayed the complete hierarchy of ECFC (Figure II.6B) when examined clonally, and the distribution of proliferative potential was similar to BCECs that were resident in corneal endothelium prior to plating (Figure II.4B). Taken together, we propose that a single cell clonogenic assay is an alternative method to stringently and directly quantitate corneal endothelial progenitor cell residence.

Consistent with their great proliferative ability, HPP-ECFCs in corneal endothelium display high levels of telomerase activity (Figure II.5). The telomere length in corneal endothelium has been measured in vivo and in vitro. In human subjects, the average telomere length has been reported as 11-14kb (Amano 2003, Egan et al 1998). It should be realized that there was no statistically significant difference in the telomere length of the corneal ECs residing in the central and peripheral areas of freshly examined corneal endothelium or when corneal EC from younger and older age groups were compared (Amano 2003, Egan et al 1998, Konomi & Joyce 2007). These observations have been interpreted as evidence that the limited replicative ability of corneal ECs

displayed in vivo is not due to senescence caused by successive shortening of telomeres. In contrast, it has been reported that in vitro passaging does shorten the telomere length in cultured corneal ECs (Amano 2003, Whitehart et al 2005), which leads to replicative senescence.

Corneal endothelium is formed by migration and proliferation of neural crest-derived mesenchymal cells and plays a barrier-bump function to maintain corneal clarity. Their developmental origin, anatomical localization and physical function are completely different from vascular ECs. Thus these two types of endothelial cells would be expected to exhibit different gene expression and functional properties. We report that VE-Cadherin, PECAM1 and eNOS which are specific markers associated with human vascular ECs are not expressed in human corneal ECs at the transcriptional level (Figure II.3), although Scheef et al. (Scheef et al 2007) reported that VE-Cadherin can be expressed in murine corneal ECs. The disparity of VE-Cadherin expression might be due to the difference between these two species. While incorporation of AcLDL is an important property of vascular ECs, Elner and Chang reported that corneal EC ingest native circulating LDL (Chang et al 1991, Elner et al 1991) but not chemically modified lipoproteins such as AcLDL. Our data, consistent with these previous studies, demonstrated that corneal ECs cannot incorporate AcLDL, which might be result from the absence of expression of the scavenger receptors CD36. Perhaps most surprising, bovine corneal ECs formed capillary-like structures when plated in Matrigel which is a characteristic of vascular ECs. All

these observations indicate that although corneal ECs are not exposed to the same environment as vascular ECs in vivo, they are flexible and are able to acquire some vascular ECs properties if given appropriate stimuli in vitro. This poses an intriguing possibility that, if vascular ECs can be manipulated to display corneal ECs characteristics, they may serve as an alternative autologous cell source for repair of damaged or senescent corneal endothelium.

CHAPTER III

Human Cord Blood Plasma Can Replace Fetal Bovine Serum (FBS) for in vitro Expansion of Functional Human Endothelial Colony Forming Cells (ECFCs)

Introduction

The progenitor cells for the endothelial lineage play critical roles in vascular homeostasis and regeneration in adult subjects (Asahara et al 1997, Hirschi et al 2008, Khakoo & Finkel 2005, Kovacic et al 2008, Rafii & Lyden 2003). High proliferative potential endothelial colony forming cells (HPP-ECFCs) have been identified as endothelial progenitor cells (EPCs) with robust proliferative potential in vitro and vessel-forming ability in vivo (Ingram et al 2004, Yoder et al 2007). Additionally, recent studies reveal that the concentration of ECFCs in circulation increases after vascular ischemia, which implies a possible contribution to vascular repair (Guyen et al 2006, Huang et al 2007). Thus, ECFCs become an attractive target for new vascular regenerative therapies. However, the study of ECFCs is hampered by multiple challenges in culturing the cells.

Current protocols for in vitro expansion of ECFCs mostly depend on the presence of fetal bovine serum or fetal calf serum (FBS or FCS) in the culture medium. Our preliminary data (Grimes BR, manuscript in preparation) has indicated that a high concentration of FBS destabilizes chromosomes in ECFCs and the frequency of the diploid karyotype decreases, while tetraploid or aneuploid karyotypes increase in ECFCs cultured in increasing concentrations of

FBS (Corselli et al 2008). Moreover, we found that differential expression of some arterial and venous endothelial cell (EC) gene markers in freshly isolated arterial and venous EC becomes indistinct when these cells are plated in a culture containing a high concentration of FBS (Figure III.1). This observation suggests that FBS might modify gene expression in ECFCs, and thus may affect cell properties and functions. In addition, FBS/FCS may contain potentially harmful xenogenic compounds associated with risks of transmitting infectious agents and inducing immune reactions when used in a transplantation setting (Halme & Kessler 2006, Mannello & Tonti 2007). Based on research and clinical considerations, a well-defined, serum-reduced or serum-depleted culture medium for in vitro isolation and expansion of ECFCs is greatly needed.

Human blood derivatives have been considered as alternatives to FBS. Human autologous or allogeneic serum/plasma, human umbilical cord serum, platelet enriched plasma, platelet lysate and platelet-released growth factors have been used as supplements in culture media to promote proliferation, migration and differentiation of mesenchymal stromal stem cells (MSCs) (Bieback et al 2009, Doucet et al 2005, Kilian et al 2004, Muller et al 2006, Vogel et al 2006). Similarly, human platelet lysate (Reinisch et al 2009, Reinisch & Strunk 2009) or platelet-derived growth factors (Kilian et al 2004) are able to enhance proliferation and migration of ECs and retain their vessel-forming ability.

In this study, we describe a novel culture medium supplemented with six growth factors and 1.5% human cord plasma, to efficiently recover ECFCs from human cord blood mononuclear cells (MNCs). We call this medium serum reduced medium (SRM). We demonstrate that ECFCs can be propagated in SRM, retaining their endothelial phenotype and function. When cultured in SRM, ECFCs exhibit the complete hierarchy of proliferative potential distribution and maintain genomic stability.

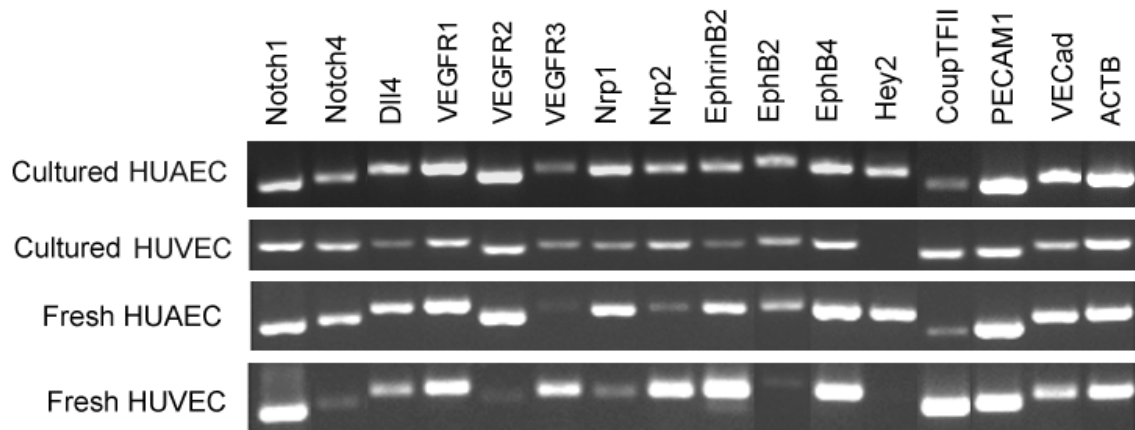


Figure III.1 The expression of arterial and venous specific genes in human umbilical artery and vein endothelial cells (HUAEC and HUVEC). The distinct arterial and venous gene expression patterns are exhibited in freshly isolated HUAECs and HUVECs, but these patterns become indistinct when cells are cultured in complete endothelial growth medium-2 (cEGM-2) containing 10% defined FBS.

Materials and Methods

Media and supplements

Human Endothelial Serum Free Medium (SFM; Invitrogen, Grand Island, NY) was supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (hrbFGF) (Invitrogen), 10 ng/ml human recombinant epidermal growth factor (hrEGF) (R&D, Minneapolis, MN), 10 ng/ml vascular endothelial growth factor 165 (VEGF₁₆₅) (R&D), 10 ng/ml VEGF₁₂₁ (R&D), 10 ng/ml stem cell factor (SCF) (R&D), 5 ng/ml stromal cell derived 1 alpha (SDF1 α) (R&D), 10 ng/ml interleukin 6 (IL6) (R&D) and 1.5% human umbilical cord plasma (HCP), to create our serum-reduced medium (SRM). As a control, human EGM-2 medium (Lonza, Walkersville, MD) was supplemented with 10% FBS (Hyclone, Logan, UT) and 1.5% penicillin/streptomycin (Invitrogen), and called complete EGM-2 medium, or cEGM-2.

Preparation of pooled HCP

Human umbilical cord blood (UCB) samples (50-100 mL) were collected in heparin-coated syringes (20 to 30 USP units of heparin/mL of blood) from healthy newborns (38- 40 weeks gestation). The Institutional Review Board at Indiana University School of Medicine reviewed and approved this study with exempt IRB status. UCB was diluted 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen) and overlaid onto Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Cells were centrifuged for 30 minutes at room temperature at 1500 rpm. After centrifugation, the mononuclear

cells (MNCs) were collected for culturing endothelial cell colonies, and the supernatant was collected for preparing human cord plasma (HCP). Subsequently, the supernatant was aliquoted and frozen at -80°C. After thawing, aliquots with the same volume from at least 20 samples were pooled and sterilely filtered through a 0.2 µm filter. The pooled HCP was then added to SRM.

Isolation and culture of UCB-derived ECFCs

MNCs were isolated and washed with DPBS. For outgrowth of ECFC colonies, MNCs either were resuspended in SRM or cEGM-2 medium. MNCs (3×10^7 /well) were seeded onto 6-well tissue culture plates precoated with Type I rat-tail collagen (BD Biosciences; Bedford, MA) and cultured as previously described (Ingram et al 2004). Spindle-shaped ECFC colonies emerged sequentially from the MNCs and the first day of ECFC colony emergence was recorded. The frequency of ECFC colonies was determined by measuring the total number of colonies in the primary culture on day 10 (as no ECFC ever emerged at a later timepoint). Subsequently, the ECFC-derived ECs were released from the primary culture dish by TrypLE™ Express (Gibco, Grand Island, NY) and replated onto 25 cm² tissue culture flasks pre-coated with Type I rat-tail collagen for subsequent passage.

Immunophenotyping of ECFC derived ECs

Early passaged (1-2) ECFC-derived ECs (5×10^4) were incubated at 4°C for 30 minutes in 100 µl of medium with varying concentrations of the primary or isotype

control antibody as outlined below, washed three times, and analyzed by fluorescence-activated cell sorting (FACS®) (Becton Dickinson, San Diego, CA). The primary antibodies we used included anti-human CD31 conjugated to phycoerythrin (PE) (BD Biosciences Pharmingen; Bedford, MA), anti-human CD34 conjugated to allophycocyanin (APC) (BD Biosciences Pharmingen), anti-human CD144 conjugated to PE (BD Biosciences Pharmingen), anti-human CD146 conjugated to PE (BD Biosciences Pharmingen), anti-human cKIT conjugated to APC (eBioscience; San Diego, CA), anti-human VEGFR1 conjugated to PE (BD Biosciences Pharmingen), anti-human VEGFR2 conjugated to fluorescein isothiocyanate (FITC) (BD Biosciences Pharmingen), anti-human VEGFR3 conjugated to APC (R&D), anti-human Nrp1 conjugated to PE (Miltenyi Biotec; Auburn, CA), anti-human Nrp2 (R&D) conjugated to Alexa Fluor 647 (Molecular Probes, Eugene, OR), anti-human CD14 conjugated to PE (BD Biosciences Pharmingen), anti-human CD45 conjugated to FITC (BD Biosciences Pharmingen), anti-human AC133 conjugated to APC (Miltenyi Biotec) and anti-human CXCR4 conjugated to FITC (BD Biosciences Pharmingen). For negative controls, we used directly conjugated mouse IgG isotypes (BD Biosciences Pharmingen).

FISH analysis of interphase cells

ECFC derived ECs after 30 days of culture from initiation (representing approximately 15 population doubling) were collected and resuspended in hypotonic solution, followed by fixation in 3:1 w/v methanol acetic acid and

dropped onto microscope slides, as previously described (Grimes et al 2009). Probes specific to the centromeres of chromosome 17 (CEP 17, Spectrum Green) and chromosome X (CEP X, Spectrum Orange) were purchased from Abbott Molecular Inc. (Des Plaines, IL). Following probe hybridization and washes, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). The signals obtained with the 17 and X probes indicating at least 200 interphase nuclei per experiment were counted using a Leica DM5000B fluorescent microscope (Leica Microsystems; Bannockburn, IL). Images were captured with a Spot RTKE camera (Diagnostic Instruments; Sterling Heights, MI) at 100x magnification.

Single cell clonogenic assays

Early-passage (1-2) ECFC-derived ECs were plated at one cell per well into 96 well plates pre-coated with Type I rat-tail collagen in 200 μ l of cEGM-2 medium. Cells were cultured at 37°C in a humidified incubator with 5% CO₂. Media were changed every five days. After 14 days of culturing, cells were fixed with 4% paraformaldehyde (Sigma; St. Louis, MO) in phosphate-buffered saline for 30 minutes at room temperature, then washed twice, stained with 1.5 μ g/ml DAPI, and examined for the growth of ECs. Those wells containing two or more cells were identified as positive for proliferation under a fluorescent microscope at 10x magnification. Wells containing fewer than 50 cells were counted by visual inspection with a fluorescent microscope at 40x magnification. For those wells

with more than 50 cells, colonies were imaged and cell number quantified using an Image J1.36v program (Wayne Rasband, NIH).

In vivo matrix implantation assays

Early-passaged (3-5) ECFC-derived ECs (2×10^6 cells/mL) were suspended in a 1.5 mg/mL collagen-fibronectin matrix as previously described (Yoder et al 2007). Aliquots (250 μ l) were pipetted into wells of 48 well plates, allowed to polymerize at 37°C for 30 minutes, and covered with 500 μ l of culture medium for overnight incubation at 37°C, in 5% CO₂. After 18 hours of ex vivo culture, cellularized matrices were implanted into the flanks of 6- to 8-week-old NOD/SCID mice as previously described (Yoder et al 2007). After 14 days, mice were euthanized and the grafts were harvested, fixed in formalin-free zinc fixative (BD Biosciences), paraffin embedded, bisected, and sectioned (6 μ m) for analysis by histology and immunohistochemistry (n=6).

Histology and Immunohistochemistry

Sections were stained as previously described (Yoder et al 2007). Paraffin-embedded tissue sections were deparaffinized and then either directly stained with hematoxylin and eosin (H&E) or immersed in retrieval solution (Dako, Carpinteria, CA) for 20 minutes at 90-99°C. Slides were incubated at room temperature for 30 minutes with anti-human CD31 (clone JC70/A, Abcam), followed by a 10-minute incubation with LASB2 link-biotin and streptavidin-HRP

(Dako), then developed with DAB (Vector, Burlingame, CA) solution for 5 minutes.

Statistical Analysis

Results are shown as the mean \pm the standard error of the mean (SEM). Data were analyzed with ANOVA; parametric test and significant differences were set at $P < 0.05$. All analyses were performed using GraphPad InStat software (GraphPad Software Inc. La Jolla, CA).

Results

Isolation and expansion of human UCB ECFCs

We and others have successfully isolated human ECFC-derived EC colonies from low-density MNCs in umbilical cord blood by utilizing cEGM-2 medium (Au et al 2008a, Ingram et al 2004, Melero-Martin et al 2007). To evaluate whether SRM is able to promote ECFC outgrowth and proliferation, we compared these two culture media directly. MNCs from the same donor were divided into portions with half of them cultured in SRM and the rest in cEGM-2. The first ECFC colonies were detected after 4.35 ± 0.25 days in SRM, compared to 6.10 ± 0.38 days in cEGM-2 ($p < 0.001$, Figure III.2A). No difference was observed in the frequency of ECFCs recovered on day 10 under these two conditions (Figure III.2B). Colonies in SRM displayed a cobblestone appearance with variations in colony size, which indicated their heterogeneous proliferative abilities (Figure III.2C) as previously reported (Ingram et al 2004).

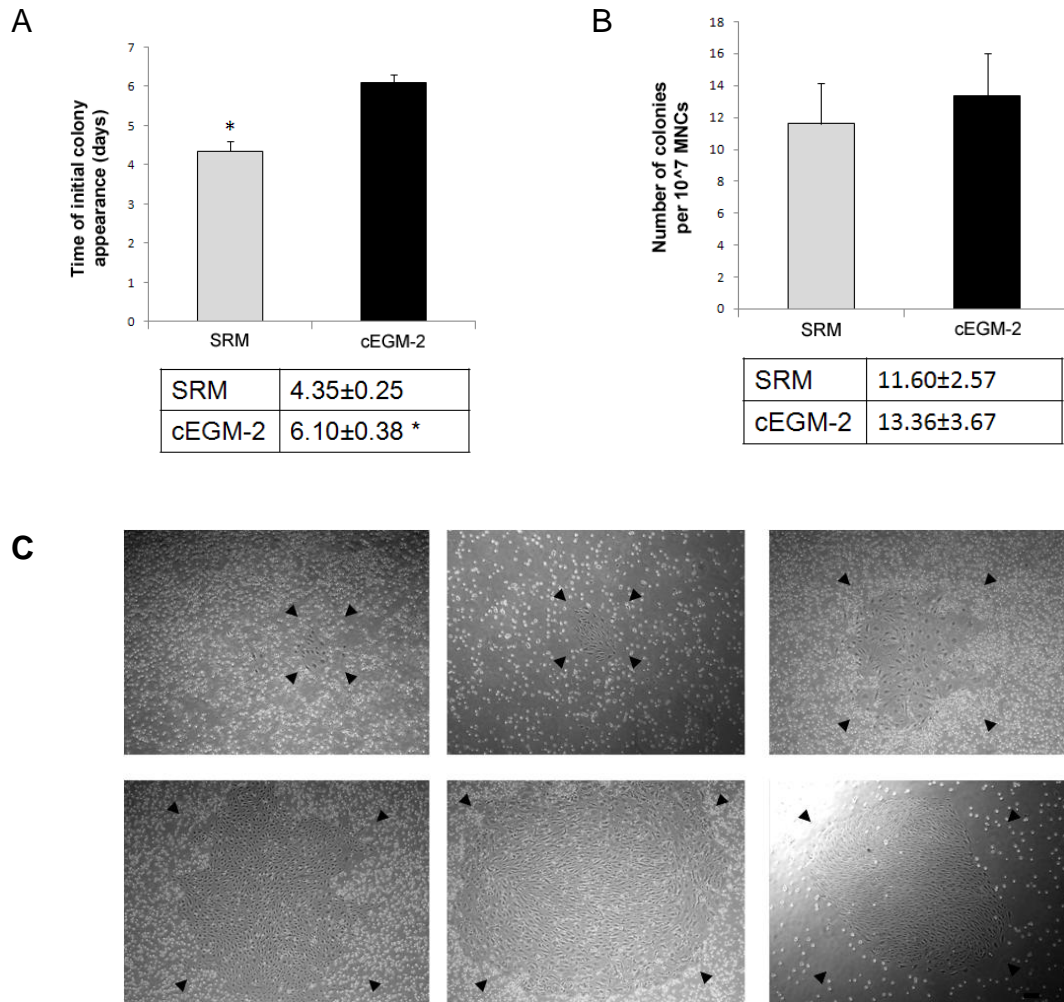
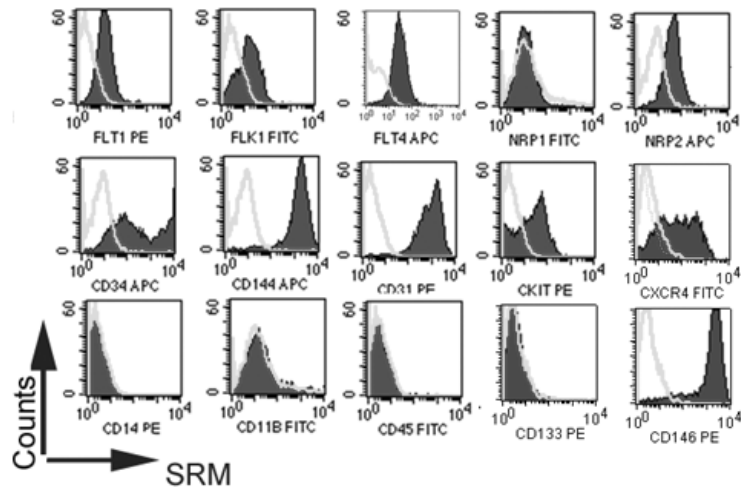


Figure III.2 Isolation of human cord blood ECFC-derived EC colonies from UCB MNCs by using SRM. (A) Time of initial ECFC derived EC colonies emerged from MNCs after culture initiation in SRM and cEGM-2. Results represent the mean number of days before initial EC appearance \pm SEM ($n = 23$, $*P < 0.001$). (B) Number of ECFC-derived EC colonies outgrown per 10^7 MNCs after 10 days of culture initiation in SRM and cEGM-2. Results represent the average number of EC colonies \pm SEM ($n = 23$). (C) Representative photomicrographs of individual human ECFC-derived EC colonies from UCB in SRM. Scale bar represents 100 μ m.

Phenotypic characterization of human UCB ECFCs

The ECFC colonies expanded and formed an endothelial monolayer in both types of culture media conditions. Immunophenotyping of the endothelial monolayer (Figure III.3) revealed that ECs cultured in SRM expressed endothelial cell-surface antigens CD31, CD34, CD144, CD146, VEGFR1, VEGFR2 and VEGFR3, which was similar with those cultured in cGEM-2. However, the expression of cKIT (the receptor of SCF) and CXCR4 (the receptor of SDF α 1) was higher in ECs in SRM than in cEGM-2. This observation can be the result of the addition of hSCF and hSDF α 1 in the endothelial culture medium (Broudy et al 1994, Volin et al 1998). Most importantly, the EC colonies cultured in SRM did not express the hematopoietic cell surface antigens CD11b, CD14, CD45 or AC133, which indicates that the HCP-supplemented culture environment was devoid of hematopoietic cell contamination.

A



B

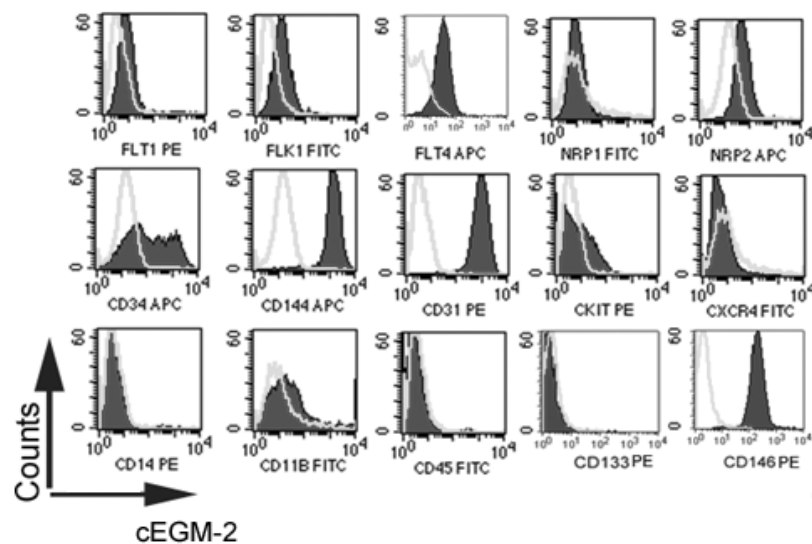


Figure III.3 Phenotypic analysis of human cord blood ECFC-derived ECs cultured in SRM. Immunophenotyping of EC from the cultured monolayer derived from human cord blood ECFC in SRM (A) and cEGM-2 (B) by fluorescence cytometry. Similar to cells grown in cEGM-2, the ECs cultured in SRM expressed CD31, CD34, CD144, CD146, Flt-1, Flk-1, Flt-4, and Nrp2 but not CD45, CD14, CD11b or AC133. Moreover, the expression of cKit and CXCR4 was detectable in ECs grown in SRM, but not in cEGM-2.

Clonogenic ability and genomic stability maintained in human UCB ECFCs

A complete hierarchy of ECFC in human peripheral and UCB derived ECs, based on proliferative and clonogenic abilities, has been previously identified (Ingram et al 2004). To determine whether such a proliferative hierarchy was also present in the ECs cultured in SRM, a single-cell clonogenic assay was performed. After single cells were plated in culture, some cells didn't divide; while other cells divided and formed colonies of different sizes comprised of varying cell numbers. The frequency of single cells undergoing division was similar between samples cultured in SRM and those in cEGM-2 (28.10 ± 21.04 vs 34.30 ± 20.89 , respectively). Moreover, the entire hierarchy of ECFCs, composed of high proliferative (HPP)-, low proliferative (LPP)-ECFC, endothelial-cluster and non-dividing mature ECs, was exhibited in ECs cultured in SRM (Figure III.4).

FISH with probes specific to the centromeres of chromosome 17 and chromosome X was utilized to detect chromosomal stability in the ECFC progeny. In preliminary experiments, we found that sequential passage of ECFC in cEGM-2 resulted in a decrease of the frequency of the diploid (2n) karyotype and an increase of tetraploid (4n) and aneuploid karyotypes, which indicated that a high concentration of FBS may disturb the chromosome stability (Grimes BR, manuscript in preparation). Here we showed that 1.5% HCP-supplemented SRM better maintains genetic stability in ECFC progeny than cEGM-2 (Figure III.5). After 30 days of culturing, 85.45% of all cells grown on SRM retained their diploid karyotype, which was higher than cells grown in cEGM-2 (73.54%). Conversely,

the frequency of the tetraploid karyotype was noticeably higher in cEGM-2 (25%) than in SRM (7.27%).

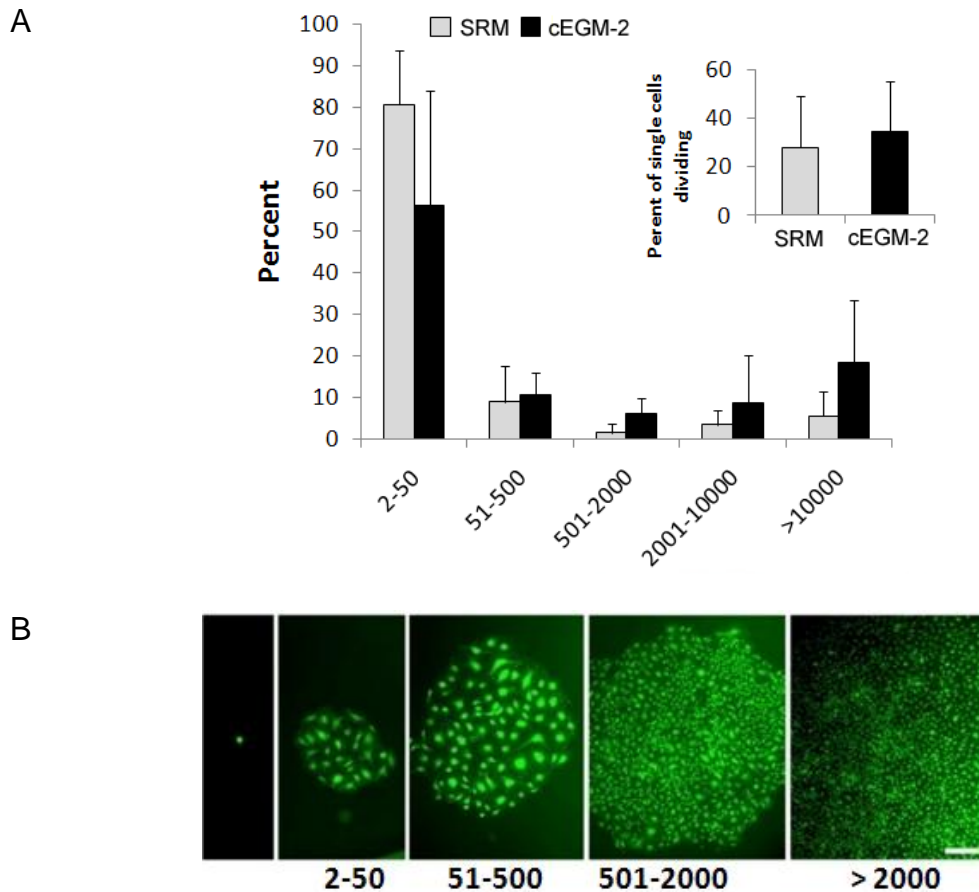


Figure III.4 Quantitation of the clonogenic and proliferative potential of single ECs derived from human cord blood cultured in SRM. (A) The distribution of colony sizes, where colonies were derived from single ECs grown in individual wells after 14 days of culture. The complete hierarchy of ECFCs was present in ECs cultured in SRM; that is similar to those grown in cEGM-2. Inset chart is the percentage of single ECs dividing at least once after growing 14 days in culture. No statistical difference in this frequency was observed between cells grown in SRM and those in cEGM-2. (n = 5) (B) Representative photomicrographs of EC colonies with varied sizes derived from single ECs. Scale bar represents 100 μ m.

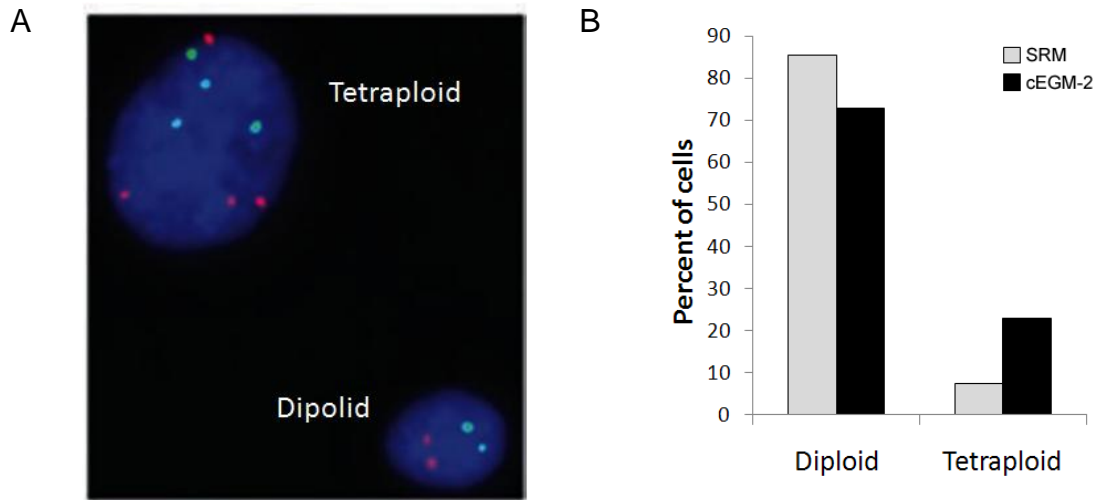


Figure III.5 Genomic stability in human cord blood ECFCs cultured in SRM. (A) Fluorescence in situ hybridization (FISH) analysis of ECs using centromere probes specific for the X chromosome (red) and chromosome 17 (cyan). Nuclei are stained with DAPI (blue). Normal (diploid) female cells display two X chromosomes (red spots) and two chromosome 17s (cyan spots). Tetraploid female cells display four X chromosomes (4 red spots) and four chromosome 17s (4 cyan spots). (B) FISH analysis of > 200 ECs after 30 days of culture initiation revealed a higher diploid content in cells grown in SRM than those in cEGM-2. Chromosomally aberrant cells (tetraploid) were detectable at a frequency up to 25% in cells cultured in cEGM-2.

In vivo formation of chimera blood vessels

We (Yoder et al 2007) and others (Au et al 2008a, Au et al 2008b, Melero-Martin et al 2008, Melero-Martin et al 2007) have demonstrated that human UCB-derived ECFCs possess the potential to form de novo blood vessels when suspended in a collagen-fibronectin matrix or Matrigel and implanted subcutaneously into immunodeficient mice. To test the in vivo vessel-forming ability of UCB ECFCs cultured in SRM, the same methods were employed. After 14 days of carrying implants containing ECFC cultured in SRM or cEGM2, the mice were euthanized; the grafts were harvested, and analyzed for human or murine blood vessel formation.

H&E staining revealed the formation of human microvessels perfused with murine red blood cells in the graft, indicating human vessel anastomoses with the surrounding murine vasculature (Figure III.6A). To further verify the human origin of these vessels, an immunohistochemistry study with a specific anti-human CD31 antibody was conducted and the results are shown on Figure III.6A. Thus, ECFC progeny cultured in SRM can also form functional human-murine chimeric vessels in a short-term xenograft model of blood vessel formation similar to cEGM2 media cultured cells. Quantification of human microvessels that carry murine erythrocytes (Figure III.6B) showed that there was no statistical difference between the implanted cells cultured in these two culture media (SRM vs CEGM-2 is 28.48 ± 14.86 vs. 14.73 ± 6.69 vessels/mm²). Furthermore, the size

distribution of these functional microvessels formed by ECFC cultured in SRM was similar to those cultured in cEGM-2 (Figure III.6C).

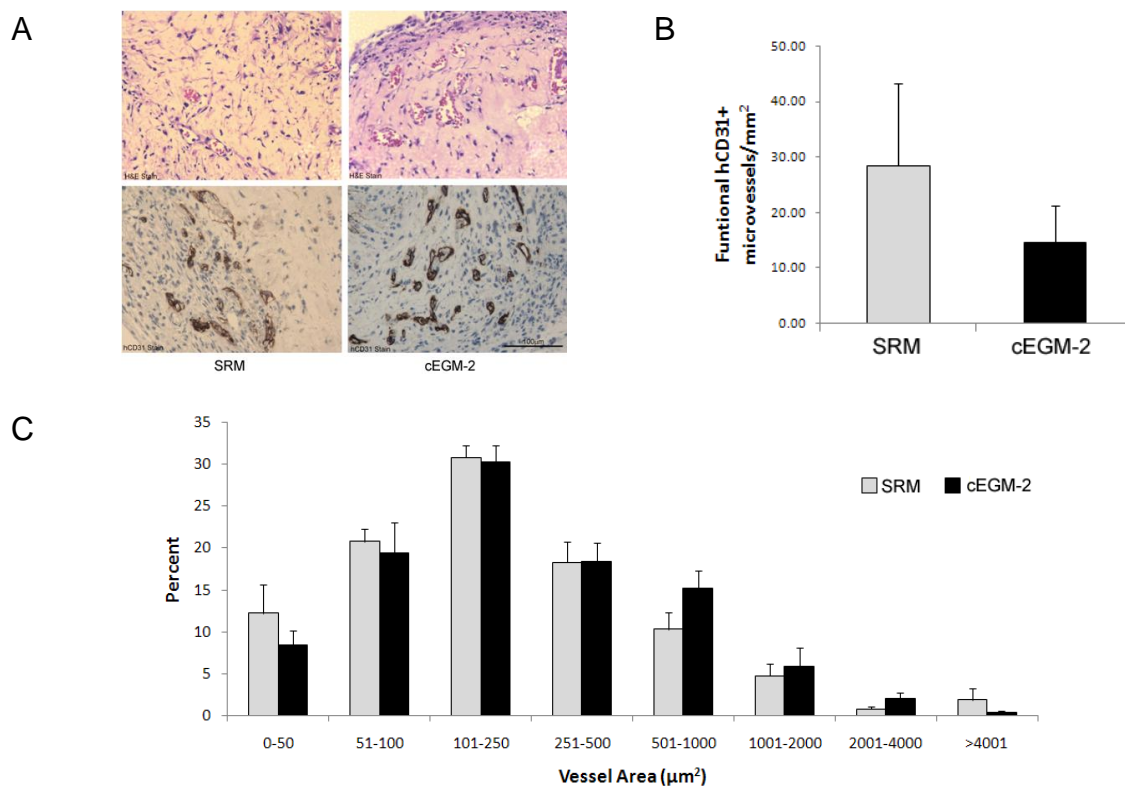


Figure III.6 Human cord-blood-derived ECFC cultured in SRM demonstrate the potential to form functional microvessels in immunodeficient mice. (A) H&E staining indicates microvessel formation in collagen-fibronectin gel after 14 days of implantation in NOD/SCID mice. Anti-human CD31 staining further confirms the human origin of these vessels. Scale bar represents 100 μm . (B) The number of vessels formed by human cord-blood-derived ECFCs and perfused with murine red blood cells per mm^2 in the gel after 14 days of implantation. (n=6) (C) The size distribution of the microvessels formed by human-cord-blood ECFCs. These data indicate that there is no difference in the vessel-forming abilities of ECs cultured in SRM versus those in cEGM-2.

Discussion

We have demonstrated a novel method that, for the first time, allows us to efficiently isolate and expand human ECFCs in vitro with a low concentration of HCP (1.5%) with no bovine serum additives. In SRM, human ECFC colony yields remain quantitatively similar as those in cEGM-2. Cells display robust proliferative ability in vitro and vessel forming capacity in vivo. Most importantly, cells in SRM are noted proved to be more genomically stable than those cultured in cEGM-2 as indicated by FISH analysis.

A recent study illustrated that human platelet lysate (HPL) can replace FBS for large-scale propagation of human ECFCs (Reinisch et al 2009). This study supported the idea that human blood products can maintain endothelial cell growth in vitro similar to the support provided to MSC (Bieback et al 2009, Schallmoser et al 2007, Schallmoser et al 2008). Human endothelial serum-free medium (SFM), the basal medium in this culture system, is commercially available and when supplemented with 20 ng/ml recombinant hbFGF and 10 ng/ml recombinant hEGF is reported by the manufacturer to support the isolation and long-term culture of human umbilical cord artery and vein endothelial cells (HUAECs and HUVECs) and human dermal microvascular endothelial cells (HMVECs) (Invitrogen manuals). However, that culture medium formulation did not support human cord blood ECFC outgrowth in our pilot assay. Consequently, we had to consider addition of other factors.

An increasing number of cytokines and chemokines have been demonstrated as displaying proangiogenic properties. Among them, bFGF, EGF, VEGF, SCF, SDF1 α and IL6 have been extensively studied for their effects on angiogenesis (Ahmed & Bicknell 2009, Coultas et al 2005, Fan et al 2008, Gerritsen et al 2003, Heidemann et al 2004, Murakami & Simons 2008, Piao et al 2009). Thus, we added these cytokines to the basal media separately or in combination to examine whether they could promote ECFC emergence from human low-density MNCs. We found a mixture of growth factors with added 1.5% HCP to maximally promoted ECFCs to emerge from MNCs and propagate (data not shown).

While 1.5% HCP substituted for 10% FBS in our studies, we were unable to isolate and expand the ECFC in the absence of HCP. This indicates that some yet-unidentified factors reside in the cord blood plasma that are critical for endothelial cell survival and proliferation are still missing. Therefore, it is very important to attempt to define such components, as that will ultimately allow the establishment of a completely defined, serum-depleted culture system.

Additionally, the molecular mechanisms underlying the interaction of these factors supporting ECs growth need to be further investigated, to help us fully understand the biology of ECs.

Emerging research debates whether human serum is better for maintaining genomic stability than FBS. Some studies suggest that autologous serum may favor chromosomal stability as compared to FBS (Schallmoser et al 2008,

Shahdadfar et al 2005), while others indicate that chromosomal stability is independent of the serum source (Dahl et al 2008, Meza-Zepeda et al 2008). Our prior study (Grimes, BR manuscript in preparation) and current observation support the opinion that genomic stability of ECFC may be related to serum concentration instead of serum source. Our investigation illustrated that a high concentration of FBS (10%) led to a higher frequency of abnormal tetraploid and aneuploid forms in ECFCs during 30 days of tissue culture, compared to a low a concentration of FBS (2%) or HCP (1.5%)(Grimes, BR manuscript in preparation). One possible explanation for a serum effect on maintenance of chromosomal stability, is that a high concentration of serum speeds cell division and results in cytokinetic failure at binucleated intermediates, producing tetraploidy; or it contributes to chromosome segregation errors, increasing aneuploidy (John 1981, Lingle et al 2005, Shahdadfar et al 2005). Thus, serum-replacement (like well-defined serum derived recombinant proteins and growth factors) supplemented or serum-depleted culture media may need to be established to safely culture cells for eventual human therapeutic interventions.

As mentioned earlier, human arterial and venous ECs cultured in cEGM-2 exhibited modified arterial and venous gene expression patterns compared to freshly isolated ECs. We examined arterial and venous gene expression profiles in cells cultured with SRM. Similarly, we found some genes were upregulated and some were downregulated even after 7 days of culture from initiation (Figure III.7). This indicates that ECs can express different gene patterns when plated in

vitro, which is not surprising. In vivo, vascular endothelial cells line the inner layer of blood vessels; they are stabilized by mural cells (pericytes and smooth muscle cells) and the extracellular matrix, and are exposed to various oxygen tension and hemodynamic forces. However, in vitro, they were placed on collagen I coated plates filled with cEGM-2/SRM and were cultured under static conditions, which are totally different from an in vivo microenvironment. Therefore, a big challenge in studying ECFCs is to develop a new strategy for expanding them, while minimizing any disturbances in their phenotypical and functional properties.

Manipulating culture conditions to maintain undifferentiated status in embryonic stem cells, hematopoietic stem cells, neuronal stem cells, as well as MSC, has been extensively studied (Ludwig et al 2006, Mannello & Tonti 2007, Moon et al 2006, Reynolds & Rietze 2005). In sharp contrast, such studies on ECFCs are scarcely found. This is partially due to the paucity of knowledge regarding how endothelial stem/progenitor cells differentiate to various lineages: arterial ECs, venous ECs and capillary ECs. However, such studies are necessary and important, as they can lay the foundation for new therapies for vascular disorders.

In summary, we report here the development of a novel formula of endothelial cell culture medium that is free of FBS and is supplemented with 1.5% HCP for expansion of human cord blood ECFCs in vitro. In creating this medium, we have also demonstrated that ECFCs grown on this medium retain their phenotype and

function, as well as their proliferative and neoangiogenic properties. This work contributes to the development of a complete serum-free culture system for ECFCs that we and others (Reinisch & Strunk 2009) believe will be required for future use as a human cell therapeutic.

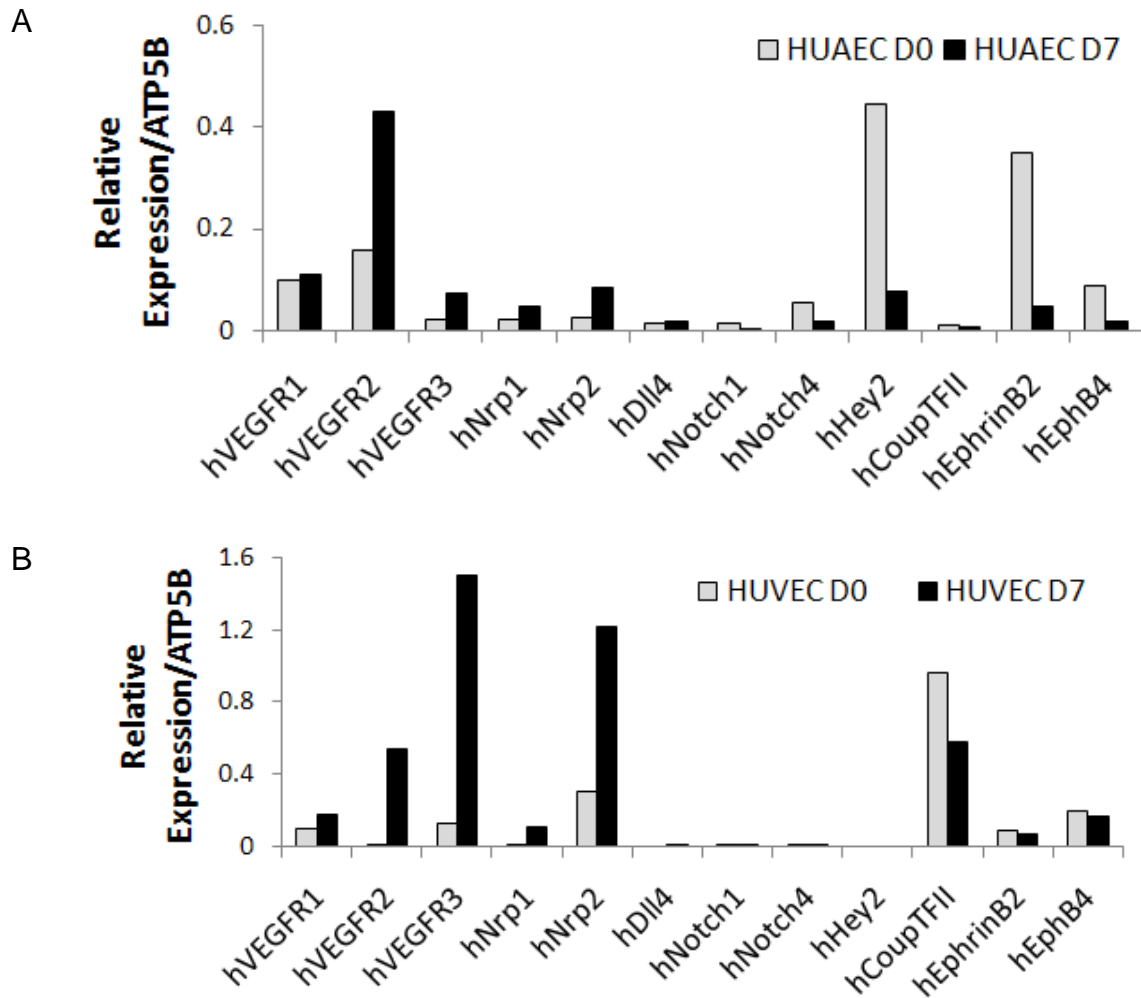


Figure III.7 The expression of arterial and venous specific genes in HUAECs and HUVECs is measured by quantitative PCR. (A) Compared to freshly isolated HUAECs (D0), the arterial gene expression pattern was modified in HUAECs plated in SRM for 7days (D7). **(B)** HUVECs cultured in SRM for 7 days adopt a gene expression profile which was different from freshly isolated HUVECs.

CHAPTER IV

Dose-dependent Effects of Vascular Endothelial Growth Factor₁₆₅ (VEGF₁₆₅) and Notch Ligand Delta like 1 (Dll1) on in vitro Differentiation of Human Cord Blood Derived Endothelial Colony Forming Cells (ECFCs)

Introduction

The vascular system is systematically organized into arteries, veins and capillaries. It has been believed that the endothelial cell (EC)-derived primary capillary plexus differentiates into arteries and veins due to the influence of hemodynamic forces. However, recent studies reveal that arterial and venous specification is at least partially governed by genetic factors. In murine embryos, arterial- and venous-specific molecular markers are detectable when the primary capillary plexus is formed, before circulation is established. Numerous molecules and signaling pathways have been described that participate in arteriovenous (AV) differentiation. Arterial endothelial cells express high levels of Notch1, Jagged1, Delta like ligand 4 (Dll4), EphrinB2 (EDNB2), Neuropilin 1 (Nrp1) and Hey2 (Duarte et al 2004, Krebs et al 2000, Lawson et al 2002, Shutter et al 2000, Villa et al 2001, Wang et al 1998, Zhong et al 2001). On the other hand, venous endothelial cells are characterized by high level expression of EphB4, Neuropilin 2 (Nrp2), and COUP TFII (NR2F2) (Wang et al 1998, You et al 2005).

The Notch signaling pathway is an evolutionarily conserved pathway that is involved in a variety of developmental processes. Notch family members and Notch ligands are expressed throughout early vascular development and later

restricted in arteries. The expression of Notch1, Notch4, Jagged2, Dll1 and Dll4 are specifically expressed in arterial endothelium. The role of these molecules in arterial specification has been demonstrated during murine embryogenesis (Adams & Alitalo 2007, Alva & Iruela-Arispe 2004, Rossant & Howard 2002, Shawber & Kitajewski 2004, Siekmann et al 2008). VEGF is one of the most potent and ubiquitous vascular growth factors that affect many aspects of endothelial cell biology. Recently, VEGF-A has been found to interact with the activated Notch pathway to determine and maintain arterial endothelial cell fate.

We have successfully isolated circulating ECFCs from human umbilical cord blood. Using single cell clonogenic assays and functional assays, a hierarchy of ECFCs has been identified (Ingram et al 2004). Human cord blood ECFCs form microvessels in immunodeficient mice after subcutaneous implantation in collagen-fibronectin gels (Yoder et al 2007). Thus, human ECFCs derived from cord blood display properties consistent with stem/progenitor cells for the endothelial lineage. However, whether these cells can be directly specified to an arterial or venous fate has so far not been addressed. We hypothesized that human cord blood derived ECFCs are not committed to either an arterial or venous fate and are able to be differentiated into arterial ECs when given appropriate stimuli.

Materials and Methods

Media and supplements

Human Endothelial Serum Free Medium (SFM; Invitrogen, Grand Island, NY) was supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (hrbFGF) (Invitrogen), 10 ng/ml human recombinant epidermal growth factor (hrEGF) (R&D, Minneapolis, MN), 10 ng/ml vascular endothelial growth factor 165 (VEGF₁₆₅) (R&D), 10 ng/ml VEGF₁₂₁ (R&D), 10 ng/ml stem cell factor (SCF) (R&D), 5 ng/ml stromal cell derived 1alpha (SDF1 α) (R&D), 10 ng/ml interleukin 6 (IL6) (R&D) and 1.5% human umbilical cord plasma (HCP), to create serum-reduced medium (SRM).

Isolation and culture of human umbilical cord blood (UCB) derived ECFCs

Human UCB samples (50-100 mL) were collected in heparin-coated syringes from healthy newborns (38-40 weeks gestation). The Institutional Review Board at Indiana University School of Medicine reviewed and approved this study with exempt IRB status. UCB was diluted 1:1 with DPBS (DPBS; Invitrogen) and overlaid onto Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). Cells were centrifuged for 30 minutes at room temperature (RT) at 1500 rpm. Mononuclear cells (MNCs) were isolated and washed with DPBS. For outgrowth of ECFC colonies, MNCs were resuspended in SRM. 3×10^7 MNCs were seeded onto each well of 6-well tissue culture plates precoated with Type I rat-tail collagen (BD Biosciences; Bedford, MA) and cultured as described in Chapter III. ECFC colonies appeared around 4 days of culture and were identified as spindle-

shaped in appearance. After approximately 10 days of culture, the ECFC-derived ECs were released from the culture dish by TrypLE™ Express (Gibco, Grand Island, NY) and replated onto 25 cm² tissue culture flasks pre-coated with Type I rat-tail collagen for subsequent passage. Characterization of human UCB ECFC derived ECs was conducted using monoclonal antibodies and fluorescence-activated cell sorter (FACS) analysis as described in Chapter III.

Isolation and culture of human umbilical artery endothelial cells (HUAECs) and human umbilical vein endothelial cells (HUVECs)

Human umbilical cords were obtained from healthy newborns (38-40 weeks gestation). The Institutional Review Board at the Indiana University School of Medicine reviewed and approved this study with exempt IRB status. In each cord, either an umbilical artery or umbilical vein was cannalized, rinsed twice with DPBS supplied with 4.8 mM sodium pyruvate (Invitrogen), and then infused with 100 µg/mL Liberase Blendzyme2 (Roche Applied Science, Indianapolis, IN) and 4.8 mM sodium pyruvate supplemented DPBS. After 14 minutes of incubation at 37°C, detached ECs were eluted into a 50 mL Falcon tube and centrifuged at 1300 rpm for 10 minutes. The cell pellet was resuspended and cells were washed with DPBS twice. The ECs were then stained with anti-human CD31 and CD45 antibodies (BD Biosciences Pharmingen, San Diego, CA) and were sorted in a BD FACS Aria cell sorter (BD Biosciences) to isolate a CD31⁺CD45⁻ population of purified ECs using morphologic phenotypic and functional validation as previously described (Ingram et al 2005b).

Immobilization of Delta1^{ext}-IgG protein

Delta1^{ext}-IgG protein (the extracellular domain of Dll1 fused to the Fc domain of human IgG1) was provided by Dr. Irwin D. Bernstein (Fred Hutchinson Cancer Research Center, Seattle, WA) (Delaney et al 2005, Varnum-Finney et al 2003). Non-tissue culture-treated plates were coated with decreasing concentrations of Delta1^{ext}-IgG (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/mL) or the same concentration of human IgG (Sigma-Aldrich, St. Louis, MO), diluted in PBS together with 5 µg/mL fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan). The plates were incubated overnight at 4°C, washed with PBS 3 times, and further incubated with 2% bovine serum albumin (BAS) dissolved in PBS at 37°C for 1 hour. Thereafter, the plates were washed with PBS 3 times and ready for plating cells.

In vitro arterial specification of human UCB derived ECFCs

Early-passage (2-4) human cord blood ECFCs were cultured in SRM with 50 or 100 µg/mL of rhVEGF₁₆₅, or plated in Delta1^{ext}-IgG coated non-tissue culture plates (as described above) with SRM for 14 days to induce an arterial EC phenotype. Media were changed every 2 days.

RNA isolation and conventional/quantitative RT-PCR

Total cellular RNA was extracted with an RNeasy Micro extraction kit (Qiagen, Valencia, CA) as described by the manufacturer. RT reactions were performed using an Omniscript RT Kit (Qiagen). Conventional PCR was conducted by using

Go Tap Flexi DNA Polymerase (Promega, Madison, WI) according to the manufacturer's instructions. The primer sequences are shown in Table IV.1.

Gene	Forward	Reverse	Tm	Product size (bp)
DLL4	TATTGGGCACCAACTCCTTC	ACATAGTGGCCGAAGTGGTC	55	349
NOTCH1	GGCCAGAACTGTGAGGAAAA	GCAGTAGAAGGAGGCCACAC	57	327
NOTCH4	TCTCCCTCTCCATTGACACC	TGGAAGCACTCGTTGACATC	55	323
HEY2	TGGGGAGCGAGAACAATTAC	GCACTCTCGGAATCCTATGC	55	329
VEGFR1	AGTTTAAAAGGCACCCAGCA	ACGAGCTCCCTTCCTTCAGT	55	362
VEGFR2	GAGGGACTTGGA CTGGCTTT	GATTTGAAATGGACCCGAGA	55	302
VEGFR3	TGAACATCACGGAGGAGTCA	TCAGGCTTGTTGATGAATGG	55	337
NRP1	GAAAAATGCGAATGGCTGAT	AATCCGGGGGACTTTATCAC	53	335
NRP2	CAAACACTGTGGGAACATCG	TGTCCAGCCAATCGTACTTG	55	338
EFNB2 (C)	TTATTTGCCCCAAAGTGGAC	CCTGGTTGATCCAGCAGAAC	55	347
EPHB4	GAGCTGTGTGGCAATCAAGA	ACTTTGCAGACGAGGTTGCT	55	345
COUPTFII	AACACATCGAGTGCGTGGT	CAGGTACGAGTGGCAGTTGA	55	311
PECAM1	GCAAAATGGGAAGAACCTGA	ACAGTTGACCCTCACGATCC	55	316
VECAD	TGGACAAGGACACTGGTGAA	TCTTGCAGAGTGACCAGCAC	55	382
ACTB	GCCAGCTCACCATGGATGAT	GTCTCAAACATGATCTGGGTC	57	388

Table IV.1: Primers used for conventional RT-PCR

The PCR cycle profile was 94°C for 5 minutes; 94°C for 30 seconds, 53 or 57°C (depending on the different primers) for 30 seconds, 72°C for 45 seconds, and 32 cycles with a final 72°C for 7-minute. PCR products were added to wells in a 2% agarose/ethidium bromide gel and exposed to electrophoresis current. Migrating bands were photographed under UV light.

Quantitative PCR was performed using FastStart Universal SYBR Green Master 2x (Rox) (Roche). Amplification was carried out in an ABI 7500 (Applied Biosystems, Foster City, CA), using its default program. The relative standard curve of each gene amplification was first generated to determine the amplification efficiency (Eff). ATP5B was used as a housekeeping gene expression reference. To compare gene expression levels among HUAEC, HUVEC and ECFCs, results were presented as the ratio of each gene to ATP5B expression. For rhVEGF₁₆₅ or Delta1^{ext-IgG} induction, gene expression levels in non-treated cells at Day 0 were considered as controls. Results were expressed as a fold change (in logarithmic scale) in comparison to the control. The quantitative analysis was performed according to Pfaffl's method (Pfaffl 2001).

The primer sequences are shown in Table IV.2.

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta\text{Ct target (control-treated)}}}{(E_{\text{ref}})^{\Delta\text{Ct ref (control-treated)}}}$$

E_{target} : amplification efficiency of target gene
 E_{ref} : amplification efficiency of reference gene
 $\Delta\text{Ct target (control-treated)} = \text{Ct}_{\text{target,control}} - \text{Ct}_{\text{target,treated}}$
 $\Delta\text{Ct ref (control-treated)} = \text{Ct}_{\text{ref,control}} - \text{Ct}_{\text{ref,treated}}$

Gene	Forwards	Reverse	Eff
VEGFR1	GCTTCTGACCTGTGAAGCAA	CTCGTGTTCAAGGGAGTGGT	1.987
VEGFR2	GAACATTTGGGAAATCTCTTGC	CGGAAGAACAATGTAGTCTTTGC	1.917
VEGFR3	AGACAAGAAAGCGGCTTCAG	TTGGGAGTCAGGGTGTGC	1.860
NRP1	GTTGTGTCTTCAGGGCCATT	AATCCGGGGGACTTTATCAC	2.036
NRP2	TCTGCGCTACGAGATCTTCA	GTGCAGTCCAAGTTGTGTGG	1.862
DLL4	GACCACTTCGGCCACTATGT	TTGCTGCAGTAGCCATTCTG	1.967
NOTCH1	CTTCAATGACCCCTGGAAGA	GAAGTGGAAGGAGCTGTTGC	1.950
NOTCH4	CTGCTGCTGCTGCTATGTGT	GTCAGGAACTGGCACGTCT	1.833
HES1	TGCTTCACTGTCATTTCCAGA	GAAAGTCTGAGCCAGCTGAA	1.958
HEY2	CTTGTGCCAACTGCTTTTGA	TCATGAAGTCCATGGCAAGA	1.912
EFNB2	TCTTTGGAGGGCCTGGAT	CCAGCAGAACTTGCATCTTG	1.986
EPHB4	TTTGGCTCCTTCGAGCTG	GGCCAAGATTTTCTTCTGGTG	1.880
COUPTFII	CCAAGAGCAAGTGGAGAAGC	TCCACATGGGCTACATCAGA	1.988
ATP5B	CCACTACCAAGAAGGGATCTATCA	GGGCAGGGTCAGTCAAGTC	1.960

Table IV.2: Primers used for quantitative RT-PCR

Western Blot

Protein extracts were prepared as described previously (Yang et al 2009), electrophoresed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with anti-human CoupTFII (R&D), anti-human Hes1 (Abcam, Cambridge, MA), anti-human Hey2 (Abcam) and anti-human β -actin (Abcam). All signals were detected by enhanced chemiluminescence.

Implantation of human cord blood derived ECFCs into NOD/SCID mice

Cellularized gel implants were cast as previously described (Yoder et al 2007). Cultured ECFCs (2.4×10^6 cells/mL) were suspended in a solution containing 1.5 mg/mL rat-tail collagen I (BD Biosciences), 100 μ g/mL human fibronectin (Chemicon), 1.5 mg/mL sodium bicarbonate (Sigma), 25 mM HEPES (Cambrex), 10% FBS, 30%SFM, pH-adjusted to 7.4. Then 250 μ L of the cell suspension was pipetted into one well of a 48-well tissue culture plate, allowed to polymerize at 37°C for 30 minutes, and covered with 500 μ L SRM for overnight incubation at 37°C, in 5% CO₂. Gels were implanted into the flanks of anesthetized 6- to 9-week-old NOD/SCID mice. After 14 days, the mice were sacrificed; the grafts were excised and analyzed by histology and immunohistochemistry (n=6) as previously described (Yoder et al 2007).

Histology and Immunohistochemistry

Zinc-fixed, paraffin-embedded tissue sections (6 μ m) were deparaffinized and then either directly stained with hematoxylin and eosin (H&E) or immersed in a retrieval solution (Dako, Carpinteria, CA) for 20 minutes at 95°C to 99°C. Vector M.O.M. Immunodetection kit (Vector laboratories, Burlingame, CA) was then utilized according to the manufacturer's instructions. Sections were stained for anti-human CD31 (clone JC70A, Abcam) and Cy3 conjugated anti-mouse smooth muscle α actin (α SMA) (clone 1A4, Sigma). Purified class- and species-matched immunoglobulins (BD Pharmingen, San Jose, CA) were used for isotype controls. Sections were incubated with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) and fluorescein streptavidin or streptavidin-horseradish peroxidase (HRP) (Vector laboratories), followed by development with 3,3-diaminobenzidine (DAB) solution (Vector laboratories). All the slides were mounted using 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probe, Eugene, OR) as a nuclear marker and then analyzed by visual inspection under 40x magnification. Images were acquired using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using Metamorph 6.1 software (Universal Imaging System Corp, Westchester, PA).

Statistical Analysis

Results are expressed as mean \pm the standard error of the mean (SEM) for the study variables. Comparison of gene expression in the 3 types of ECs was

assessed by One-way ANOVA with a Tukey post test, performed using GraphPad InStat version 3.00 (GraphPad Software, La Jolla, CA). The change of gene expression after rhVEGF₁₆₅ or Dll1 induction in ECFC were assessed by Student's paired t-test. The vessel number and size distribution were evaluated by Students unpaired t test. A statistically significant difference was set at $P < 0.05$.

Results

Arterial and venous endothelial gene expression patterns are present in human cord-blood ECFCs

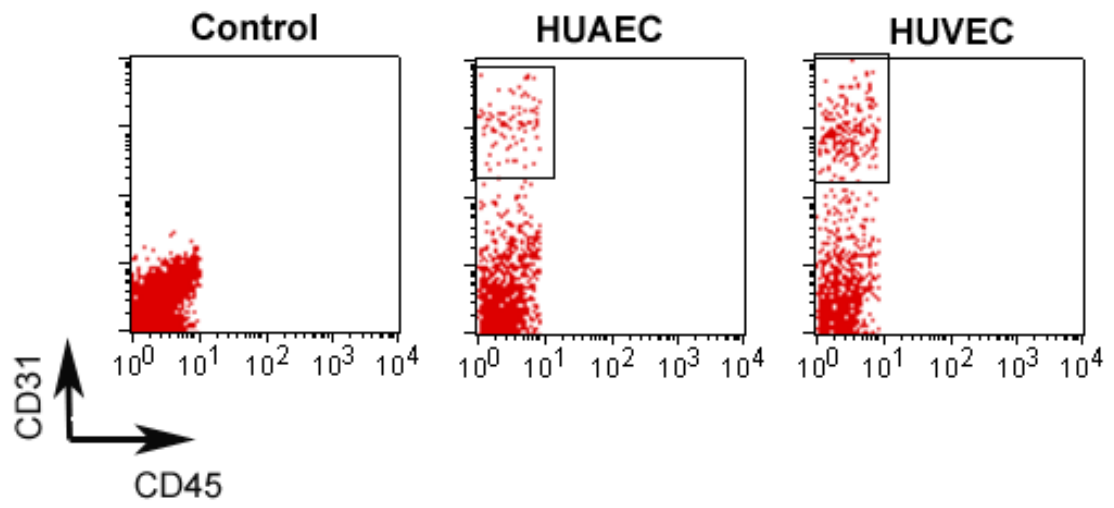
Prior studies in chick, zebrafish and mice have suggested that many molecules are involved in arterial-venous determination during embryonic development (Table I.1). Moreover, global gene expression profiling revealed both arterial and venous gene expression patterns in cultured human vascular endothelial cells (Chi et al 2003). However, it is unknown to what extent these in vitro identified arterial and venous EC gene expression profiles reflect in vivo expression patterns. Thus, we directly evaluated gene expression in freshly isolated HUAECs and HUVECs.

HUAECs and HUVECs were harvested from umbilical cords and successively sorted into a purified CD31+CD45- EC population (Figure IV.1A). Subsequently, cells were lysed and PCR was performed. As shown in Figure IV.1A, freshly isolated HUAECs and HUVECs displayed distinct arterial/venous EC gene expression patterns, which were consistent with published data (Chi et al 2003, Swift & Weinstein 2009). Dll4 and Notch4 were more abundant in HUAECs, but Notch1 predominated in HUVECs. For VEGF receptors, VEGFR2 (KDR) and NRP1 were expressed mainly in HUAECs, while VEGFR3 (FLT4) and NRP2 mRNA were present in greater amounts in HUVECs. In particular, the important arterial EC gene marker Hey2 was predominately expressed in HUAECs; while the critical venous EC gene marker CoupTFII was enriched in HUVECs.

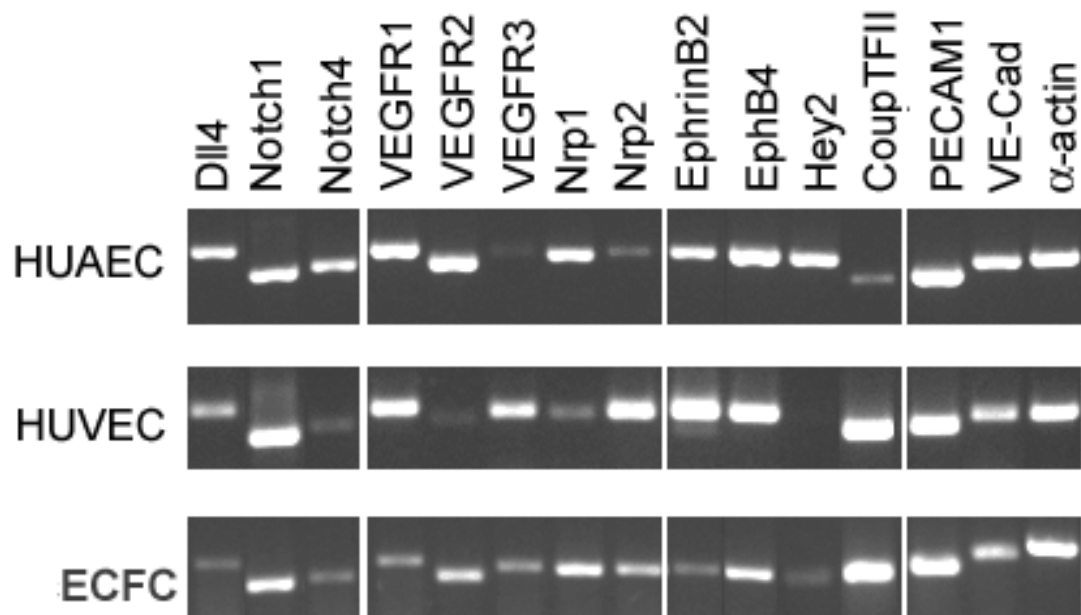
We next examined the expression of these genes in cultured human cord blood ECFCs. Of interest, ECFCs were able to exhibit both arterial and venous EC gene expression patterns, which were not weighted toward either an arterial or venous identity. Additionally, HUAECs, HUVECs and ECFCs displayed several typical EC surface markers such as PECAMI (CD31) and VE-Cadherin (VE-Cad), which confirmed their endothelial identity.

We further quantified arterial and venous EC gene expression in HUAECs, HUVECs and ECFCs by utilizing qRT-PCR (Figure IV.1C). The expression of VEGFR2, Dll4, Notch4 and Hey2 was restricted in HUAECs, while NRP2 and CoupTFII were expressed preferentially in HUVECs. The transcriptional level of EphrinB2 was significantly higher in HUAECs than HUVECs; expression of EphB4 was higher in HUVECs than HUAECs. Therefore, in this study, Dll4, Notch4, Hey2 and EphrinB2 were assigned as arterial endothelial cell gene markers, and EphB4 and CoupTFII as venous endothelial cell gene markers. It is noteworthy that human cord blood ECFCs were able to express low levels of both arterial and venous endothelial cell gene markers, and thus are not committed in vitro to either an arterial or venous fate.

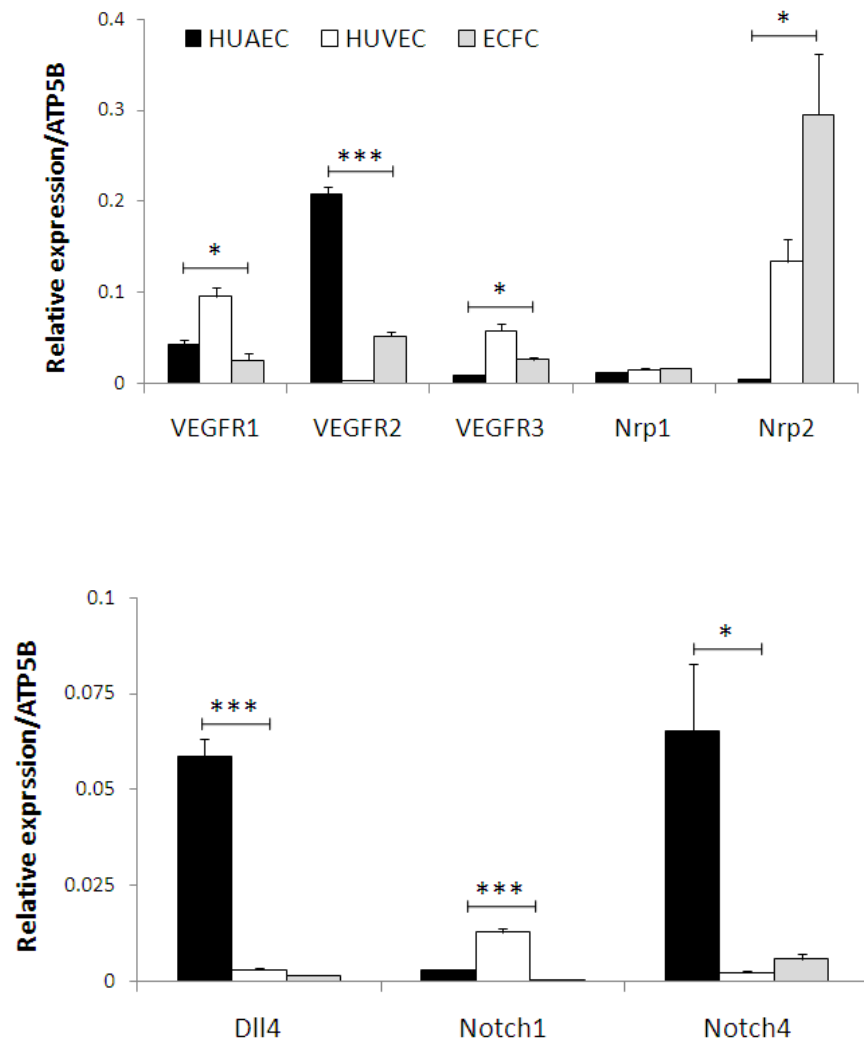
A



B



C



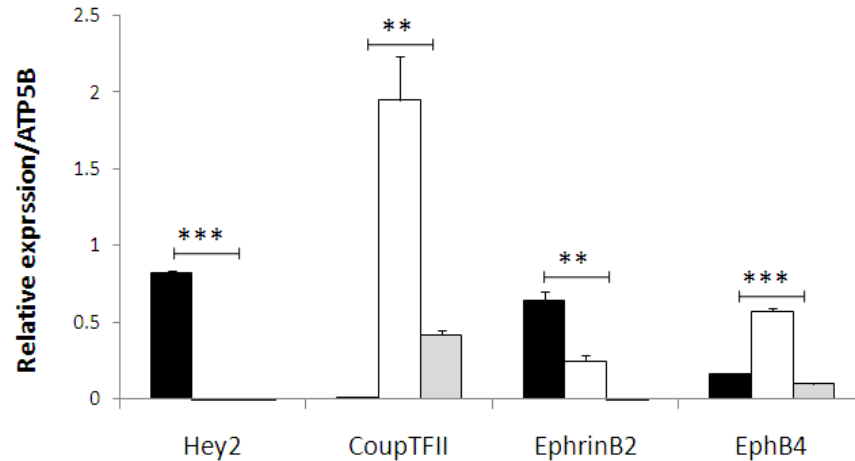
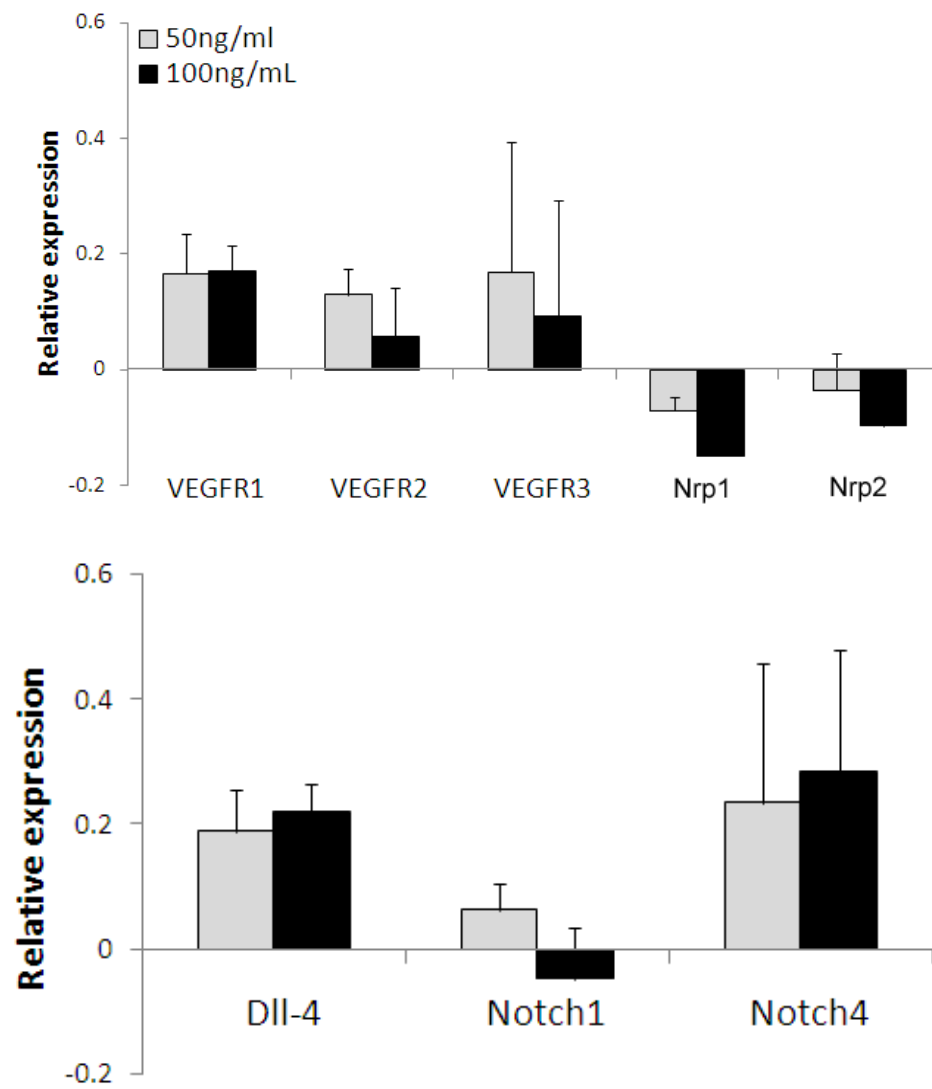


Figure IV.1 The expression of arterial and venous endothelial cell genes in freshly isolated HUAEC, HUVEC and human cord blood derived ECFCs. (A)

Representative flow-cytometric analysis of freshly isolated HUAECs and HUVECs stained with isotype controls and monoclonal antibodies against human CD31 and CD45. The CD31+CD45- population was sorted as purified endothelial cells. (B) Distinct arterial and venous gene expression patterns are exhibited in freshly isolated HUAECs and HUVECs using conventional PCR. Human cord blood derived ECFCs present both arterial and venous gene expression profiles. Moreover, three types of cells all express endothelial-cell gene markers PECAM I and VE-Cadherin. (C) Quantitative PCR further confirms the distinct gene expression patterns in freshly isolated HUAECs and HUVECs. Human cord blood derived ECFCs are able to express both arterial and venous genes, suggesting the arterial or venous fate is not determined in this cell population. The mRNA levels in all panels are expressed in ratio to a housekeeping gene ATP5B. n = 5. *P < 0.05; **P < 0.01 and ***P < 0.001 by One-way ANOVA with a Tukey post test.

Dose-dependent and time-sensitive effects of rhVEGF₁₆₅ on induction of an arterial endothelial gene expression pattern in human cord blood ECFCs

VEGF is an important upstream factor that regulates arterial differentiation during embryogenesis and throughout life (Lawson et al 2002, Visconti et al 2002). We next determined whether rhVEGF₁₆₅ is capable of inducing an arterial endothelial cell gene expression pattern in human cord blood ECFCs in vitro. Early passage (2-4) of pooled human cord blood ECFCs (n = 5) were cultured in the presence of rhVEGF₁₆₅ over 14 days. Arterial and venous endothelial cell gene expression patterns were examined throughout the progression of stimulation by quantitative RT-PCR (data not shown). This revealed that the arterial endothelial cell gene marker EphrinB2 was significantly upregulated after 14 days of culture with 50 ng/mL of rhVEGF₁₆₅. In contrast, the venous endothelial cell gene markers Coup TFI and EphB4 were not significantly affected nor were expression of the other arterial endothelial cell gene markers Dll4, Notch4 and Hey2 (Figure IV.2). These observations suggest that an arterial endothelial cell gene expression profile is not completely induced under rhVEGF₁₆₅ stimulation in vitro.



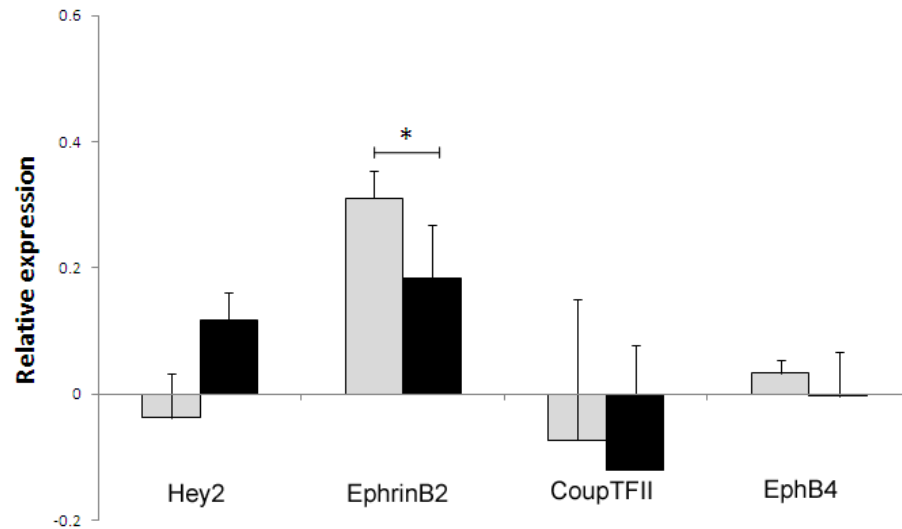


Figure IV.2: The alteration of arterial and venous endothelial cell gene expression in human cord-blood-derived ECFCs' response to 14 days of rhVEGF₁₆₅ stimulation. Q-RT-PCR analysis shows upregulation of expression of some arterial endothelial cell gene markers (VEGFR2, Dll4, Notch4,). IN particular, EphrinB2 mRNA significantly increases upon stimulation with 50 µg/mL of rhVEGF₁₆₅, while the mRNA expression of the venous gene marker CoupTFII decreases. Expression levels are presented as a fold change (in logarithmic scale) compared to baseline levels, which are normalized using ATP5B as a housekeeping gene. The expression at Day 0 in non-treated cells acts as baseline. n = 5. *P < 0.05 by paired Student's t-test.

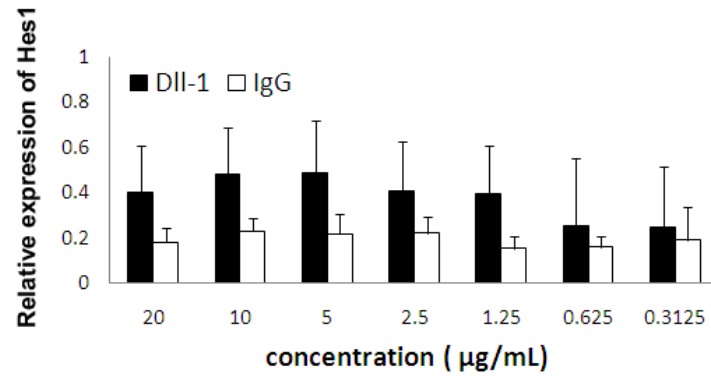
Dose-dependent and time-sensitive effects of Dll1 on induction of an arterial endothelial gene expression pattern in human cord blood ECFCs in vitro

Developmental studies indicate that activation of Notch signaling is located downstream of VEGF stimulation and further drives an arterial endothelial cell gene expression pattern in endothelial cells. We decided to test whether an arterial endothelial cell gene expression pattern could be induced in human cord-blood ECFCs directly via activated Notch signaling. In this study, the engineered Notch ligand, Delta1^{ext-IgG}, consisting of the extracellular domain of Dll1 fused to the Fc portion of human IgG, was used to activate Notch signaling. An enzyme-linked immunosorbent assay was utilized to confirm that the concentration of ligand coated on the tissue culture plate surface correlated with the amount of ligand bound as previously described (Delaney et al 2005). The linear relationship between the amount of immobilized ligand and activation of Notch signaling was measured by the expression of Notch downstream target genes Hes1 and Hey2 (Figure IV 3A and 3B). After 3 days of culturing, cells were harvested and the expression of Hes1 and Hey2 was examined by quantitative RT-PCR. Expression of Hes1 and Hey2 increased as the concentration of Delta1^{ext-IgG} increased. This indicated that human cord blood derived ECFCs were capable of responding to Notch ligand Dll1 stimulation.

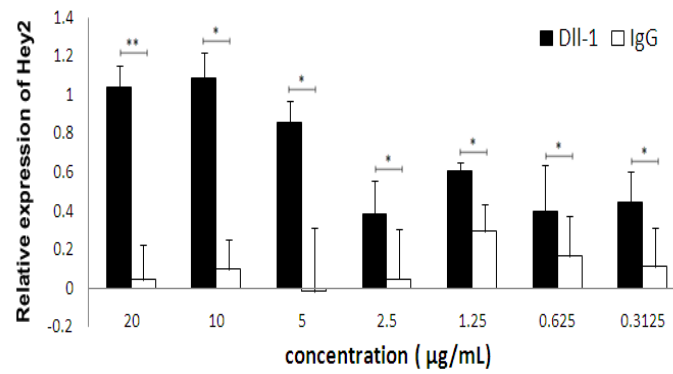
We next evaluated whether Delta1^{ext-IgG} influences arterial/venous endothelial cell gene expression patterns in human cord blood ECFCs. Early passaged (2-4)

pooled human cord blood ECFCs (n = 5) were cultured in wells coated with Delta1^{ext-IgG} at concentrations ranging from 0.312 to 20 µg/mL, or with the same concentration of human IgG as the control ligand. ECFCs displayed a Delta1^{ext-IgG} dose-dependent increase in Hes1- and Hey2-mRNA expression over 14 days of culture (data not shown). It is noteworthy that the arterial endothelial cell gene markers Hey2 and EphrinB2 were significantly upregulated after 3 days of Delta1^{ext-IgG} induction (10 µg/mL). In contrast, the expression of the venous endothelial cell gene markers CoupTFII and EphB4 were not significantly affected (Figure VI.3C). Consistently, the alteration of gene expression at the transcriptional level was confirmed at the translational level (Figure IV.3D). These results demonstrated that Notch ligand Dll1 was able to enhance the arterial endothelial cell gene marker expression in human cord blood ECFCs in vitro.

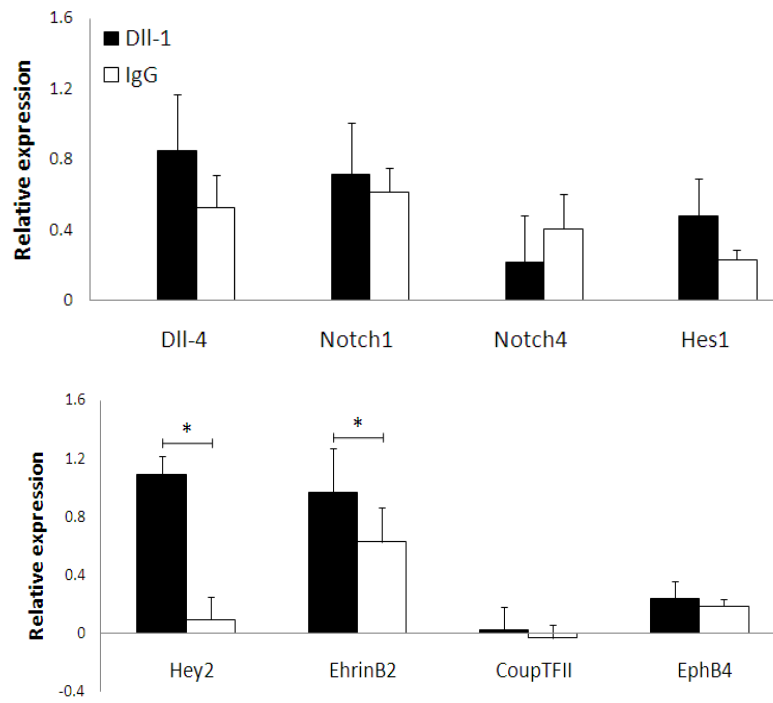
A



B



C



D

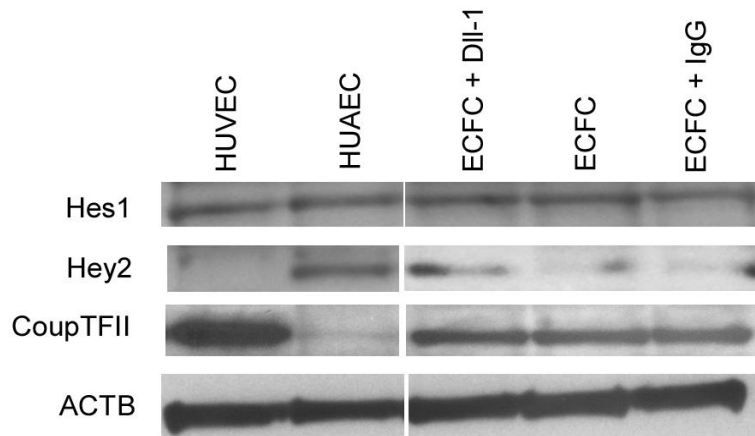


Figure IV.3 The alteration of arterial and venous endothelial cell gene expression in human cord blood derived ECFCs response to 3 days of Delta1^{ext-IgG} induction. The dose-dependent activation of endogenous Notch signaling in human cord blood derived ECFCs is indicated by Hes1 (A) and Hey2 (B) expression after 3 days of incubation in various concentrations of Delta1^{ext-IgG} using quantitative PCR. (C) Q-RT-PCR analysis shows arterial endothelial cell gene markers Hey2 and EphrinB2 are significantly increased after 3 days of stimulation with 10 µg/mL Delta 1^{ext-IgG}, whereas the expression of venous gene markers CoupTFII and EphB4 are not significantly affected. Expression levels are presented as a fold change (in logarithmic scale) compared to baseline levels and are normalized by using ATP5B as a housekeeping gene. The expression at day 0 in nontreated cells acts as baseline. n = 5. *P < 0.05 by paired Student's t-test. (D) Immunoblot analysis shows the protein level changes of Hes1, Hey2 and CoupTFII are consistent with their mRNA levels of change. HUAEC is shown as the positive control for arterial EC, and HUVEC is the as positive control for venous EC.

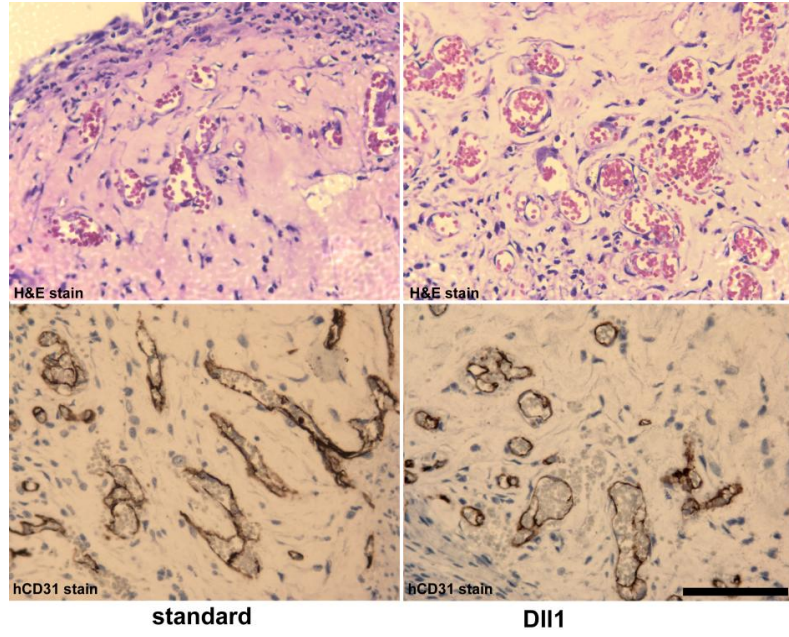
Dll1-primed human cord blood ECFCs failed to enhance the formation of arteriole-like microvessels in vivo

We further determined whether human cord blood ECFCs programmed to an arterial endothelial cell-like fate will form arteriole or arteriole-like vessels after they are implanted in vivo. ECFCs were cultured in a standard medium or treated with 10 $\mu\text{g/mL}$ of Delta1^{ext-IgG} for 3 days; then they were suspended in a collagen-fibronectin matrix and subcutaneously transplanted into immunodeficient mice. After 14 days, the mice were euthanized; the grafts were harvested and analyzed for blood vessel formation. H&E staining and immunohistochemistry analysis using a specific anti-human CD31 antibody revealed human cord blood ECFCs were able to form microvessels perfused with murine red blood cells in the graft (Figure IV.4A). Quantification of murine erythrocyte-containing human microvessels (Figure IV. 4B) showed no statistical difference between control and Delta1^{ext-IgG}-treated cell implants (28.48 ± 14.86 vs. 36.37 ± 16.37 vessels/ mm^2 , respectively). Furthermore, the size distribution of these functional microvessels formed by treated and nontreated ECFCs were similar (Figure IV.4B).

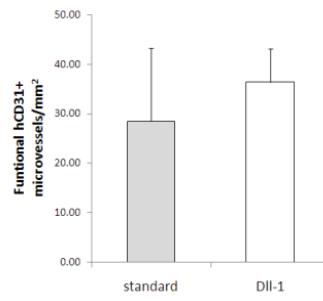
To further assess the formation of arterial-like microvessels, double-staining with anti-human CD31 and anti-mouse α smooth-muscle actin was conducted, as prior studies had demonstrated that human vessel recruitment of murine perivascular support cells in vivo (Enis et al 2005, Melero-Martin et al 2008, Melero-Martin et al 2007, Schechner et al 2000). Human ECFC-derived vessels

coated with murine α SMA-positive pericytes were indeed observed in the graft (Figure IV.4C). However, there was no statistical increase of such vessels in implants containing human cord blood ECFC primed with Delta1^{ext-IgG} compared to control nontreated cells and HUAECs (data not shown). Together, these data indicate that in vitro Delta1^{ext-IgG}-primed human cord blood ECFCs were unable to enhance arterial-like vessel formation in vivo, although they can be induced to express an arterial endothelial cell-like gene expression pattern in vitro.

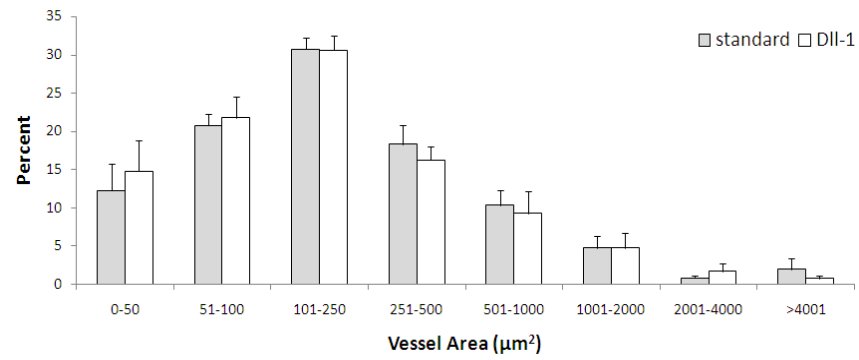
A



B



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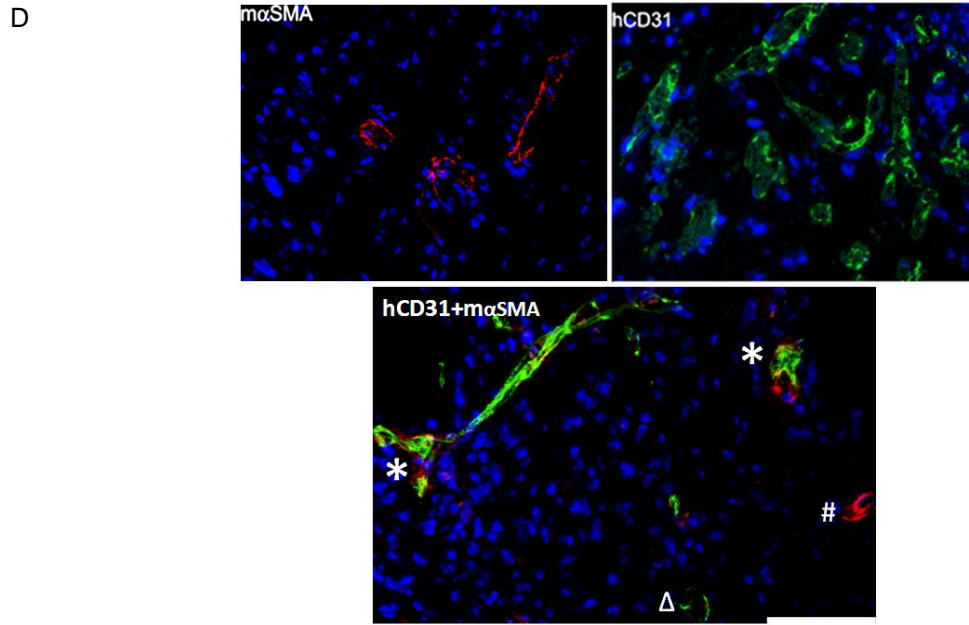


Figure IV.4 in vitro Dll1 primed ECFCs fail to display significant skewing toward arterial EC phenotype and function in vivo upon implantation. (A)

H&E staining indicates microvessel formation in collagen-fibronectin gel after 14 days of implantation. Anti-human CD31 staining further confirms the human origin of these vessels. Results suggest that human cord blood derived ECFCs cultured in the presence of Delta 1^{ext-IgG} retain vessel-forming ability in vivo.

Moreover, the number of vessels formed by human cord blood derived ECFCs and perfused with murine red blood cells per mm² in the gel demonstrate no observable difference between Delta 1^{ext-IgG} treated and nontreated samples (B).

In additions, the size distribution of both populations of microvessels is similar (C). (n = 6) (D) Vessels formed by human cord blood derived ECFCs are positive

for anti-human CD31 staining (green). Murine αSMA specifically reacts with murine pericytes (red). Double-positive staining with human CD31 and murine αSMA (*) indicates recruitment and investment of murine mural cells to human ECFC-formed microvessels, thus representing an arterial EC property. However, in vitro Dll1-primed ECFCs don't skew to display arterial EC properties when implanted in vivo; thus the number of double- positive staining vessels is not significantly increased compared to cells under standard culture conditions.

Scale bar represents 100 μm.

Discussion

Studies in chick, zebrafish and mice have revealed molecular distinctions between arterial and venous ECs, as well the signaling pathways underlying AV specification during development (Aitsebaomo et al 2008, Rocha & Adams 2009, Swift & Weinstein 2009). The most recent studies of murine embryonic stem cells (ESCs) (Lanner et al 2007, Yurugi-Kobayashi et al 2006), human multipotent adult progenitor cells (MAPCs) (Aranguren et al 2007) and human bone mesenchymal stem cell (MSCs) (Zhang et al 2008) have illustrated the potential of these stem cells to differentiate into cells displaying gene expression patterns similar to arterial and/or venous ECs. However, no reports to date have shown that human ECFCs are able to adopt an arterial or venous endothelial cell-like fate. Thus, this study is the first to demonstrate that human cord blood derived ECFCs heterogeneously express molecules that normally are restricted to arterial and venous endothelium. In vitro, exposure to Notch ligand Dll1 is sufficient to induce an arterial gene expression pattern in ECFCs. However, the in vitro Dll1-primed ECFCs, when implanted, failed to display significant skewing toward an arterial EC phenotype or function in vivo. Nonetheless, this study contributes to the understanding of possible mechanisms involved in AV differentiation in vitro and postnatal arteriogenesis in humans, which ultimately may provide potential new therapeutic strategies for human cardiovascular diseases.

Studies in zebrafish and mice have indicated that VEGF plays a critical role in determining AV specification and that the concentration of VEGF influences this determination. In murine ESCs, a high concentration of VEGF (50 ng/mL) drove arterial endothelial cell gene expression (Dll4, Notch4, Nrp1 and EphrinB2), while a low dosage of VEGF (2 ng/mL) encouraged venous endothelial cell gene expression (CoupTFII) (Lanner et al 2007). Similarly, 100 ng/mL of rhVEGF₁₆₅ could upregulate arterial endothelial cell gene marker expression in hMAPCs (Dll4, Hey2, EphrinB1 and EphrinB2) and in hMSCs (Dll4, Notch4 and EphrinB2) (Aranguren et al 2007, Zhang et al 2008). Our results consistently showed that VEGF at 50 ng/mL was sufficient to enhance expression of the arterial endothelial gene EphrinB2 only. Although the expression of Dll4 and Notch4 was increased, the change was not statistically significant. Likewise, upon VEGF stimulation, the expression of venous gene marker CoupTFII was downregulated but not significantly. These findings suggest that a high concentration of VEGF is able to induce the expression of at least one arterial endothelial cell gene in cultured human circulating ECFCs.

Notch is another critical factor in determining and promoting vascular development. Mice with deficiencies in Dll4, Notch1, RBP-Jk and Hey1/2 lose expression of arterial endothelial cell gene markers (Duarte et al 2004, Fischer et al 2004, Kokubo et al 2005, Krebs et al 2004, Uyttendaele et al 2001). Dll1 recently has been established as necessary for maintaining arterial identity during fetal development and mediating postnatal arteriogenesis in mice with

hindlimb ischemia (Limbourg et al 2007, Sorensen et al 2009). Dll1 expression in the vascular endothelium is detected in murine embryos at embryonic day 13.5 (E13.5) in arteries but not in capillaries and veins. Dll1-mediated Notch activity via Notch1 is pivotal for controlling arterial identity during fetal development (Sorensen et al 2009). Similarly, Dll1-dependent Notch signaling is required for upregulation of EphrinB2 in growing arteries (Limbourg et al 2007). In this regard, the signaling cascade (Dll-Notch/Hey2/EphrinB2) responsible for determining arterial identity, which has been extensively studied in Dll4 (Aitsebaomo et al 2008, Rocha & Adams 2009, Swift & Weinstein 2009), is now also present in Dll1. However, Dll1's influence on arterial specification in human ECFCs has not yet been addressed. In this study, we showed that human ECFCs were responsive to Dll1 stimulation in a dosage-and time-dependent manner. After 3 days of Dll1 induction (10 µg/mL), the expression of critical arterial gene markers (Hey2 and EphrinB2) were noticeably increased, which is consistent with prior studies of HUVECs where Dll4 and Notch4 were constitutively expressed (Shawber et al 2003, Williams et al 2006). On the other hand, venous endothelial cell gene markers (CoupTFII and EphB4) were not influenced under Dll1 induction, which also has been observed when Notch4 or the Notch intracellular domain (NICD) was overexpressed in HUVECs (Iso et al 2006, Shawber et al 2003). Thus, these data illustrate that Dll1 induction of arterial endothelial cell-like gene expression is independent of suppression of the venous endothelial cell-like phenotype in human circulating ECFCs cultured ex vivo.

Although an arterial endothelial cell-like gene expression pattern was able to be induced in human circulating ECFCs in vitro upon Dll1 stimulation, these ECFCs failed to display significant skewing toward an arterial EC phenotype and arterial functions in vivo. A possible reason for this is that these cells were not continuously exposed to Dll1 stimulation. It had been reported that only immobilized Dll1 can activate Notch, and soluble Dll1 inhibits Notch (Varnum-Finney et al 2000). Delta-1^{ext-IgG} was not directly supplemented in the implanted collagen-fibronectin gel in this present study. To overcome this diffusion restriction, some carriers or scaffolds will need to be employed to immobilize Dll1 in the gel in future studies. In this case, both the cell carrier and cell density should be determined to ensure that ligand-cell and cell-cell interfaces are interacting directly to induce Notch activation.

It has been reported that Bcl2-transduced HUVECs suspended in a collagen-fibronectin matrix and implanted under mouse abdominal skin were able to be remodeled into arterioles, venules and capillaries after 60 days (Enis et al 2005, Schechner et al 2000). Additionally, human ECs co-implanted with murine mesenchymal precursor cells 10T1/2 or human bone-marrow-derived MSCs were capable of giving rise to long-lasting, stable, engineered vessels and demonstrating distinct arterial and venous functional characteristics (Au et al 2008a, Au et al 2008b). These findings suggest the potential of human ECs to be differentiated into functional arterial ECs in vivo, which is consistent with our short-term implantation outcomes. In the present study, anti-human CD31 and

anti-murine α SMA staining revealed that some human ECFC-formed vessels were covered with murine periendothelial mesenchymal cells, which indicated that human ECFCs adopt an arterial endothelial cell-like phenotype in vivo. However, there was no dramatic increase in recruitment and investment of mural cells to vessels derived from Dll1-primed ECFC compared to those formed by nontreated ECFC or HUAEC (data not shown). Addition of mural cells to the ECFC may be an additional strategy to provide Notch ligand exposure to the implanted human ECFC and progeny.

In summary, we have demonstrated that human cord blood derived ECFCs are not committed to either an arterial or venous fate during in vitro culture but can be induced in vitro to display an arterial endothelial cell-like gene expression pattern after exposure to Notch ligand Dll1. However, in vitro Dll1-treated ECFCs fail to display significant skewing toward an arterial EC identity upon implantation, suggesting that in vitro priming is not sufficient for in vivo specification in our current experimental model. Our findings contribute to a greater understanding of the mechanisms underlying A/V differentiation in human ECFCs and may also provide novel insights into future therapeutic strategies for vascular regeneration in human subjects with vascular disease

CHAPTER V

Acute Myocardial Infarction in Swine Rapidly and Selectively Releases Highly Proliferative Endothelial Colony Forming Cells (ECFCs) into Circulation

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Abstract

Background: We have recently identified endothelial colony forming cells (ECFCs) in human blood and blood vessels and ECFC are elevated in patients with coronary artery disease. Since pigs are a favored model for studying myocardial ischemia, we questioned whether ECFCs also exist in swine and whether myocardial ischemia would alter the number of ECFC in circulation.

Methods and Results: ECFCs were present in circulating blood and aortic endothelium of healthy pigs. In pigs with an acute myocardial infarction (AMI) (n=9), the number of circulating ECFC was markedly increased compared to sham control pigs (15 ± 6 vs. 1 ± 1 colonies/100cc blood, $P < 0.05$). Moreover, the percentage of circulating high proliferative potential-ECFCs (HPP-ECFCs) was significantly increased following AMI induction compared to sham control ($38.4 \pm 5.8\%$ vs. $0.4 \pm 0.4\%$, $P < 0.05$) and to baseline ($38.4 \pm 5.8\%$ vs. $2.4 \pm 2.4\%$, $P < 0.05$) blood samples.

Conclusions: This is the first study to report that ECFCs are present in blood and aorta in healthy pigs and that the number and distribution of circulating ECFCs is altered following AMI. Since circulating ECFC are also altered in human subjects with severe coronary artery disease, the pig model of AMI may be an excellent preclinical model to test the role of ECFC in the pathophysiology of AMI.

Introduction

In 1997, Asahara and colleagues described a cell population derived from human circulating CD34⁺ cells that could differentiate ex vivo into cells with endothelial cell-like characteristics (Asahara et al 1997). These cells were termed endothelial progenitor cells (EPCs). Subsequent evidence suggested that these cells are derived from bone marrow, circulate in peripheral blood, and play an important role in both postnatal vasculogenesis and vascular homeostasis (Asahara & Kawamoto 2004, Khakoo & Finkel 2005, Kubota et al 2003, Miyamoto et al 2004, Rafii & Lyden 2003). However, the characterization of EPCs remains controversial. Currently, human EPCs are defined as cells that express CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR2; KDR)(Peichev et al 2000). Human EPCs and their progeny have also been shown to express the endothelial markers von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (CD31), and to ingest acetylated low-density lipoprotein (LDL) and bind to *Ulex europaeus agglutinin* I (Ingram et al 2005a). Efforts to accurately define EPCs have been complicated by the presence of circulating cells that have certain properties of endothelial cells, but have a monocytic origin, termed circulating angiogenic cells (CACs) (Rehman et al 2003), as well as mature circulating endothelial cells (CECs) (Blann et al 2005, Dignat-George & Sampol 2000). These latter cells express the S-endothelial antigen (CD146) but also express some of the same proteins as EPCs, such as CD31, VEGFR2 and vWF.

In humans, another population of EPCs, termed late outgrowth endothelial cells has been reported (Gulati et al 2003, Lin et al 2000). These are marrow-derived cells that display robust proliferative potential ex vivo. Recently, we have determined that the late outgrowth endothelial cells represent one subpopulation of circulating endothelial colony forming cells (ECFCs) (Ingram et al 2004). A complete hierarchy of ECFCs displaying varying levels of proliferative potential has been isolated and characterized in human umbilical and adult peripheral blood and as well as in a variety of blood vessels(Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004). The cord blood ECFCs express high levels of telomerase and contribute to new vessel formation when implanted in vivo (Yoder et al 2007).

Several recent reports have demonstrated that increased circulating concentrations of various populations of EPCs are correlated with reduced risk for an adverse cardiovascular outcome (Chen et al 2004, Ding et al 2007, Eizawa et al 2004, Güven et al 2006, Hill et al 2003, Loomans et al 2004, Mutunga et al 2001, Nadar et al 2005, Schmidt-Lucke et al 2005, Solovey et al 1997, Vasa et al 2001, Wang et al 2005, Werner et al 2005). However, patients with severe coronary artery stenosis that are clinically selected for a revascularization procedure also display the highest concentration of circulating late outgrowth cells (Güven et al 2006), highlighting the linked questions whether the increase of these cells represents a direct response to the occurrence of ischemia and whether they play a risk-modulating role in the context of ischemia. Since pigs

are a favored species to examine the pathophysiology of coronary artery injury and acute myocardial ischemia (AMI), we questioned whether circulating ECFCs are present in the blood of healthy pigs and whether inducing an AMI would specifically alter the circulating concentration of ECFCs.

We have determined that ECFCs are resident in the aortic endothelium of normal pigs and circulate in pig peripheral blood, however, the frequency of these circulating cells is extremely rare. The circulating ECFCs in healthy pigs display proliferative potential that is limited. In pigs that have been stressed by an induced AMI, however, ECFCs with high proliferative potential (HPP-ECFC) promptly emerge in the circulation. Thus, swine possess circulating ECFCs as do adult human subjects, and the onset of myocardial ischemia leading to infarction is accompanied by an increase in concentration of the ECFCs, with preferential mobilization of a highly proliferative subset.

Materials and Methods

Blood sample collection

Animal handling and care was performed under the recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Yorkshire domestic pigs (25-35 Kg) of mixed gender were enrolled in this study. Study animals were sedated with IM Ketamine (20 mg/kg), and atropine (0.05 mg/kg), followed by IV sodium pentothal (25 mg/kg). After intubation, anesthesia was maintained with Isoflurane (2.5%). Arterial access was obtained via the common carotid artery using a cutdown technique, and an 8F vascular sheath was used to cannulate the artery. Left heart catheterization was performed using a hockey-stick catheter (Cordis®) for coronary cannulation. A femoral artery sheath was placed to maintain continuous hemodynamic monitoring during the procedure.

AMI was created by inflating an angioplasty balloon for 45 minutes in the proximal LAD artery immediately distal to the first septal perforator. All study animals received IV amiodarone to minimize ventricular arrhythmias; amiodarone dosage was 75 mg IV bolus over 10 minutes given just prior to balloon inflation, followed by 1 mg/min infusion continued for the duration between balloon inflation and deflation. Nevertheless, sustained ventricular tachycardia and ventricular fibrillation occurred during balloon inflation in every animal (2.9 ± 1.6 episodes/procedure) and was terminated using biphasic DC cardioversion (Medtronic®). Peripheral blood was collected at baseline before AMI,

immediately post AMI and at sacrifice 7 days later (Figure V.1A). Sham control pigs underwent all operative preparation, catheter instrumentation, and three DC cardioversions but were not made ischemic (Figure V.1B).

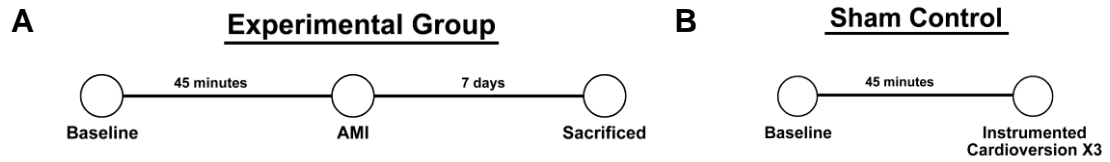


Figure V.1 Overview of experimental design. The circles indicate blood sampling times in the infarcted (A) and control sham pigs (B).

The extent of myocardial damage created by coronary occlusion was assessed by tetrazolium trichloride (TTC) staining of harvested hearts (7 days following AMI). TTC staining was performed using a standard protocol (Adegboyega et al 1997). The hearts were taken immediately after sacrifice and were sliced into 5 sections of 1.5-2.0 cm thickness along the apical-basal axis. These sections were immersed into TTC solution for 10 minutes at 37°C. Afterwards, MI areas (pale areas not staining with TTC solution) were quantified by manual tracing using NIH imaging software and were expressed as a percentage of the total left ventricular area. The mean myocardial infarction was $26 \pm 5\%$ of the whole left ventricular area in this series of 9 pigs. This model results in a decrease in left ventricular ejection fraction approximating 46% (Hou et al 2005).

Isolation and culture of porcine blood derived endothelial cells

Blood (50-100ml) was diluted one to one with Hank's/bovine serum albumin (BSA)/ethylenediamine tetraacetic acid (EDTA) solution and layered (2:1 ratio) onto Histopaque1119 (Sigma-Aldrich, St. Louis, MO). Cells were centrifuged for 30 minutes at 1800 rpm at room temperature (Beckman Coulter, Allgera Tm 6R centrifuge). Mononuclear cells (MNCs) were isolated and washed three times with endothelial cell basal medium-2 (EBM-2) (Cambrex, Walkersville, MD) supplemented with 20% porcine plasma derived serum (PDS) (Animal Technologies Inc. Tyler, TX), 2% antibiotic-Antimycotic (Invitrogen, Grand Island, NY) (complete EGM-2 medium). MNCs were resuspended in 5 ml of complete EGM-2 medium and cells were plated onto a six well plate pre-coated with mg/ml

type I rat tail collagen (BD Biosciences, Bedford, MA) and incubated at 37°C, 5% CO₂ in a humidified incubator. After 48 hours of culture, the non-adherent cells were removed and complete EGM-2 medium was added to each well. Medium was changed daily for the first seven days and then every other day until the first passage.

Colonies of endothelial cells appeared in general following 7-14 days of culture and were identified by their cobblestone appearance. Endothelial colonies were enumerated by visual inspection with an inverted microscope (Olympus, Lake Success, NY). These ECFC-derived endothelial cells were released from the culture dish by 0.25% trypsin-EDTA (Gibco, Grand Island, NY) and replated onto 25 cm² tissue culture flasks pre-coated with type I rat tail collagen for subsequent passage.

Isolation and culture of porcine aortic endothelial cells

Endothelial cells were obtained from fresh aorta pieces by gently and thoroughly scraping the vessel wall with a disposal scraper (Fisher Scientific, Pittsburgh, PA). These cells were completely transferred from the edge of scraper into six well plates filled with complete EGM-2 medium and pre-coated with type I rat tail collagen. After 48 hours of culture, the non-adherent cells were removed and complete EGM-2 medium was added to each well. Medium was changed every other day until the first passage.

Endothelial colonies appeared on day 3 of culture and kept growing to form an endothelial monolayer with typical cobblestone appearance. These aortic ECFC-derived endothelial cells were released from the culture dish by 0.25% trypsin-EDTA (Gibco, Grand Island, NY) and replated onto 25 cm² tissue culture flasks pre-coated with type I rat tail collagen for future passage.

Uptake of Dil-acetylated-low density lipoprotein (Dil-Ac-LDL)

Early passage (2-4) ECFC-derived endothelial cells were incubated with 10 µg/mL of Dil-Ac-LDL (Biomedical Technologies Inc., Stoughton, MA) in complete EGM-2 medium for 4 hours at 37°C. Cells were washed three times, stained with 1.5µg/mL of 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma), and examined by inspection through an inverted fluorescence microscope (Zeiss, Thornwood, NY) at 32x magnification.

RT-PCR for eNOS

Total cellular RNA was extracted with TRIzol (Invitrogen, Grand Island, NY) in a single-step method. RT reactions were performed using a SuperScript™ First-Strand Synthesis system (Invitrogen, Grand Island, NY). PCR was performed using the PuReTaq Ready-To-Go PCR Beads (GE Healthcare UK Limited, UK) according to the manufacturer's instructions. The primer sequences for pig eNOS included: forward, 5'- AGCGGCTGCATGACATTGAGA-3'; reverse, 5'- ATGTCCTCGTGATAGCGTTGCT-3' with a 397 base pair expected band. The primer sequences for pig GAPDH included: forward, 5'-

ACATCAAGAAGGTGGTGAAGCAGG-3'; reverse, 5'-CACCCTGTTGCTGTAGCCAAAT-3' with a 201 base pair expected band. The PCR cycle was 94°C, 5 min; 94°C 30s, 57°C 30s, 72°C 1min, and 30 cycles with a final 72°C 7 min cycle. PCR products were added to wells in a 2% agarose/ethidium bromide gel, exposed to electrophoresis current, and migrating bands photographed under UV light.

Immunophenotyping of endothelial cells

Early passage (2-4) ECFC-derived endothelial cells were stained with monoclonal antibodies including, mouse anti-rat/pig CD31 (RDI, Flanders, NJ), mouse anti-pig CD45 (SEROTEC, UK), mouse anti-pig pan tissue (BD Biosciences, Bedford, MA), mouse anti-pig SLA-DR (BD Biosciences, Bedford, MA), or mouse isotype control antibody for 30 minutes at 4°C, washed three times, and analyzed by fluorescence activated cell sorting (FACS) using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA) as previously described (Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004, Yoder et al 2007).

Single cell assays

For early passage (2-4) ECFC-derived endothelial cells, the FACS Vantage Sorter (Becton Dickinson) was used to place one cell per well into 96 well plates pre-coated with type I rat tail collagen in 200µl of complete EGM-2 medium. Cells were cultured at 37°C, 5% CO₂ in a humidified incubator. Media was

changed every five days. After 14 days of culture, the cells were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) in phosphate-buffered saline for 4 hours at room temperature, then washed twice, stained with 1.5 µg/ml DAPI, and examined for the growth of endothelial cells. Those wells containing two or more cells were identified as positive for proliferation under a fluorescent microscope at 10x magnification. Wells containing fewer than 50 cells, were counted by visual inspection with a fluorescent microscope at 10x magnification. For those wells with more than 50 cells, colonies were imaged and cell number quantified using an Image J 1.36v program (Wayne Rasband, NIH).

Statistical Analysis

Results are expressed as mean±SEM. Data were compared with nonparametric (Wilcoxon or Mann-Whitney) test and significant differences were set at the $P < 0.05$ level. All analyses were performed using InStat 2.0 software.

Results

Characterization of porcine endothelial colony forming cells (ECFCs)

We have previously reported that human peripheral blood and vessels contain a hierarchy of ECFCs (Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004). However, the existence of this ECFC hierarchy in porcine blood and vascular endothelium has not been demonstrated. To isolate ECFCs, we harvested swine peripheral blood MNCs and aortic endothelial cells. Both ECFC-derived endothelial cells (ECs) were able to form a monolayer with typical cobblestone morphology of ECs (Figure V.2A,B). Generally there were 4 ± 2 colonies growing from $8.5 \pm 4.0 \times 10^8$ blood MNCs of healthy swine ($n = 12$). The appearance of the first aortic EC colonies (3 ± 0 days) was earlier than any colonies identified from PB (11 ± 1 days); from our human studies, those colonies emerging early generally display higher proliferative potential (Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004). The PB and aortic ECFC-derived cells ingested Dil-labeled acetylated low-density lipoprotein (AcLDL) (Figure V.3A). Moreover, endothelial nitric oxide synthase (eNOS), an enzyme generally thought to be endothelial-specific, was expressed at the mRNA level in both aortic and PB ECFC-derived ECs using reverse transcription-polymerase chain reaction (RT-PCR) (Figure V.3B). To evaluate whether these cells displayed cell surface proteins with an EC phenotype, we assayed the ECFC-derived ECs for the expression of EC and leukocyte surface markers. All the ECFC-derived cells showed positive immunostaining for anti-platelet endothelial adhesion cell adhesion molecule-1 (CD31) and anti-pan-tissue antibodies, but did not express

either the common leukocyte surface marker CD45 or the SLA-DR antigen (Figure V.3C). Accordingly, the cultured cells derived from pig aorta and peripheral blood ECFCs were confirmed to be endothelial cells by both phenotypic and functional properties.

	Control group (n=3)		Experimental group (n=9)		
	Baseline	Instrumented	Baseline	Post MI	Sacrifice
MNCs (x10⁸)	8.5±4.0	2.3±1.6	3.5±1.3	4.3±0.9	3.1±0.6
ECFC	5±2	1±1*	5±2	15±6*	6±2
Colony					

Values are mean±SEM, *Instrumented vs. Post MI, p < 0.05

Table V.1 The number of MNC and ECFC colony in 100cc porcine blood

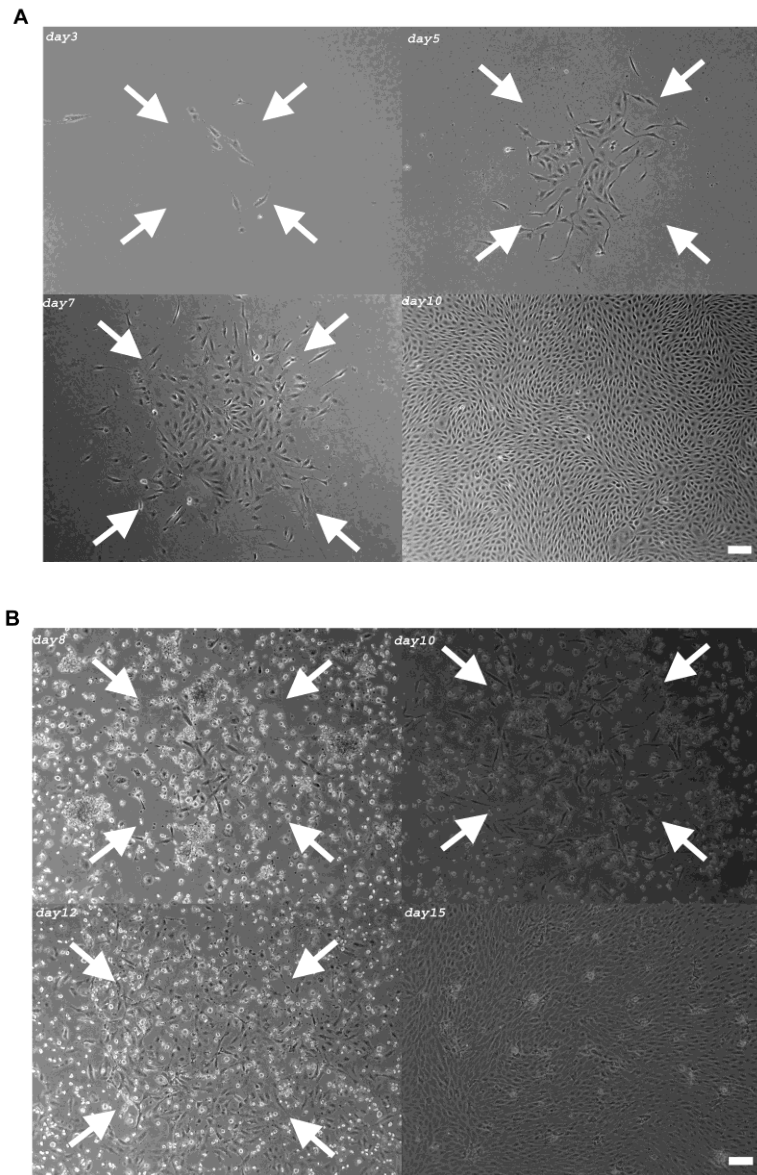


Figure V.2 The formation of porcine aortic and PB ECFC-derived endothelial colony. (A) Aortic ECFC-derived endothelial colony as it appeared on day 3, day 5, day 7 and day 10 of culture. (B) PB ECFC-derived endothelial colony from adherent MNCs emerged on day 8, day 10, day 12 and day 15 of culture. (10x magnification, scale bar represents 100 μ m)

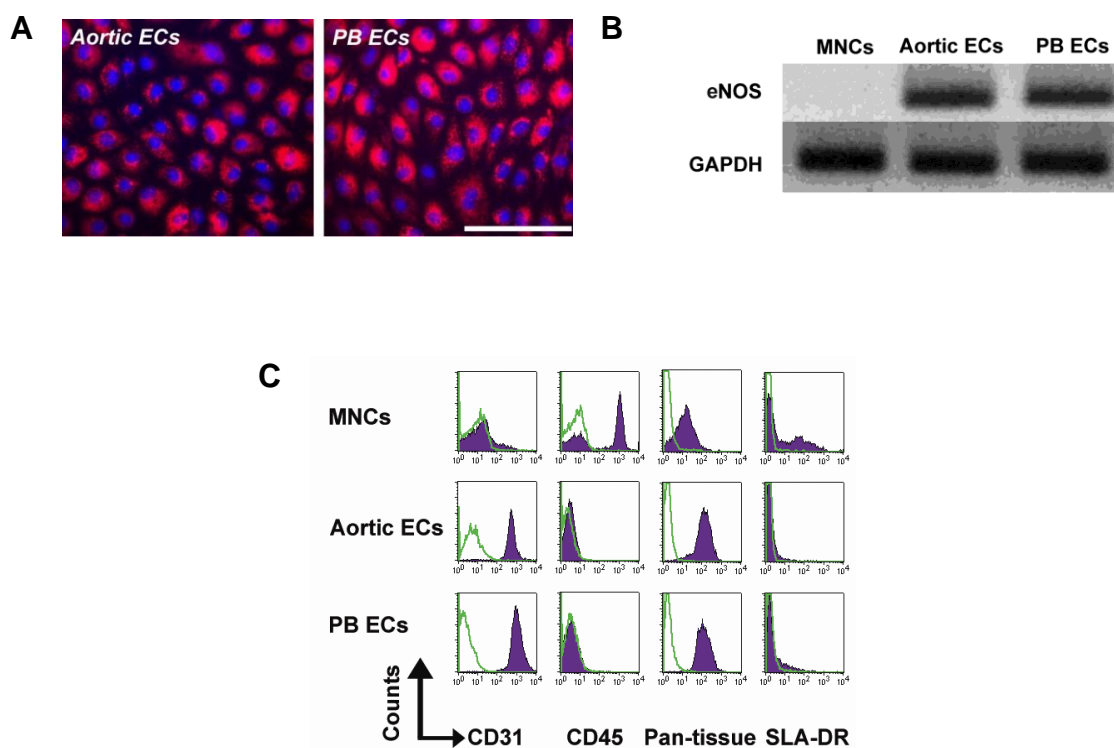


Figure V.3 Phenotypic and functional analysis of both aortic and PB ECFC-derived ECs. (A) Aortic and PB ECFC-derived ECs incorporated Dil-Ac-LDL (red) and also stained with DAPI (blue) (32x magnification, scale bar represents 100 μ m). (B) Expression of eNOS in aortic and PB ECFC-derived ECs by RT-PCR. (C) Immunophenotyping of MNCs and cell monolayers derived from aortic and PB ECFC-derived ECs by fluorescence cytometry. Both aorta and PB derived ECs expressed CD31, and Pan-tissue but not CD45 or SLA-DR antigens.

Hierarchical organization of porcine ECFC proliferative potential

Circulating EPCs have been reported to serve as a robust biomarker for predicting the severity and risk of adverse cardiovascular illnesses in human subjects (Hill et al 2003, Werner et al 2005). Though EPCs are heterogeneously defined, EPCs (including ECFCs) and CECs have been used as biomarkers. We recently developed a single cell colony-forming assay to quantitate the clonogenic potential of individual human EPCs (Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004). Using this assay we tested whether there was a hierarchy of proliferative potential in ECFC present in porcine vascular endothelium and peripheral blood. As shown in Figure V.4A, $70.4 \pm 13.9\%$ of single plated aortic ECFC-derived and $46.2 \pm 14.4\%$ of PB ECFC-derived ECs divided at least once during 14 days of culture. The hierarchy of ECFC is comprised of colonies that may be further described by the total number of progeny produced during a 14 day culture. Thus, single cells that gave rise to a colony containing more than 2000 cells were called high proliferative potential-endothelial colony forming cells (HPP-ECFC). Single cells that formed a colony containing 51 to 2000 cells were called low proliferative potential-endothelial colony forming cells (LPP-ECFC), whereas single cells that produced 2-50 cells were defined as endothelial cluster forming cells, and mature non-dividing endothelial cells retained no proliferative potential. In healthy animals at baseline, both aorta and PB contained ECFC with the full complement of dividing and non-dividing cells. The majority of these ECFC-derived colonies contained less than 2000 cells, however approximately $10.3 \pm 3.5\%$ of aortic ECFC and 1.0

$\pm 1.0\%$ of PB ECFC were classified as HPP-ECFC (Figure V.4B-C).

Photomicrographs of the size of the various endothelial colonies/clusters are shown in Figure V.4D. To determine if the HPP-ECFC-derived progeny possessed self-renewal ability (cells displaying the ability to give rise to colonies of similar or greater proliferative potential), we replated aortic and PB HPP-ECFC progeny in a single cell clonal assay. After replating, $39.6 \pm 7.0\%$ of the clonal progeny of single aortic HPP-ECFC could give rise to all subsequent stages of endothelial progenitors in addition to forming secondary HPP-ECFC (Figure V.4E-F). Similarly, $23.9 \pm 2.4\%$ of clonal progeny of single PB HPP-ECFC were able to form secondary colonies upon replating. (Figure V.4E, G). These colonies could be further replated to form tertiary colonies (data not shown). Only HPP-ECFC retained the ability to self-renew into secondary colonies with retained HPP-ECFC activity, distinguishing this colony as functionally distinct from LPP-ECFC or endothelial cluster forming cells. Thus, a hierarchy of endopoiesis was demonstrated to exist in porcine endothelial cells isolated from aorta and from circulating blood.

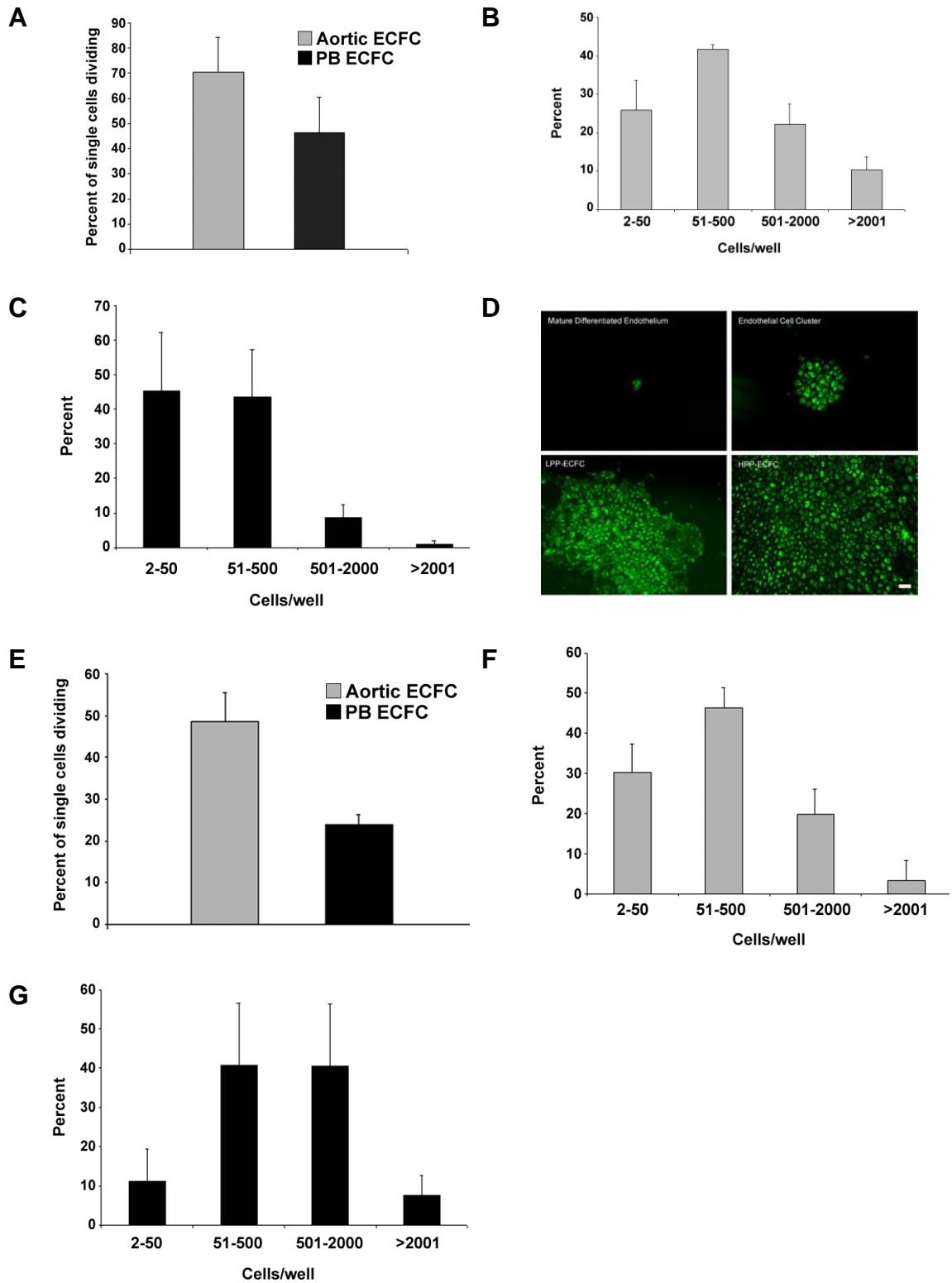


Figure V.4 Quantitation of the clonogenic and proliferative potential of single endothelial cells derived from aortic and PB ECFCs. (A) The percentage of single aortic and PB ECFC-derived ECs dividing at least once after 14 days culture. (B-C) The distribution of different size of colonies derived from single aortic (B) and PB (C) derived ECs in an individual well after 14 days of culture. (D) Representative DAPI- stained (blue) photomicrographs (10x magnification, scale bar represents 100 μ m) of the different endothelial cell clusters (< 50 cells) and colonies (> 50 cells) derived from single ECs. (E) The percentage of single endothelial cells from primary aortic and blood HPP-ECFCs that gave rise to secondary colonies after 14 days of culture. (F-G) The distribution of different sizes of secondary colonies derived from single endothelial cells, which in turn were derived from primary aortic (F) and blood (G) HPP-ECFCs. Results represent the average of at least three independent experiments from different animals. (n=5)

Alteration of the distribution of ECFCs in a porcine model of acute myocardial ischemia (AMI)

A variety of reports indicate that the concentration of EPCs and CECs changes in human subjects with acute cardiovascular injury and in those at risk for developing an adverse cardiovascular event (Chen et al 2004, Ding et al 2007, Eizawa et al 2004, Güven et al 2006, Hill et al 2003, Loomans et al 2004, Mutunga et al 2001, Nadar et al 2005, Schmidt-Lucke et al 2005, Solovey et al 1997, Vasa et al 2001, Wang et al 2005, Werner et al 2005). To examine whether the distribution of ECFCs in the circulating blood was affected during a controlled ischemic cardiovascular stress, we isolated blood derived ECFCs from pigs undergoing an experimental AMI. The AMI was created by balloon catheter inflation in the left anterior descending coronary artery (LAD) for 45 minutes. We harvested blood samples (n=9) before AMI (baseline), following AMI (post AMI) and at sacrifice 7 days later (sacrificed). Additionally, sham control pigs were anaesthetized, instrumented, and experienced 3 cardioversion procedures, but the balloon was not inflated to induce myocardial injury. There was no statistical difference in the blood MNC cell count between the 2 groups at baseline, post infarction and sacrifice (Table V.1). However, the number of circulating ECFC was noticeably higher in post AMI samples than in sham control pig blood (15 ± 6 vs. 1 ± 1 colonies/100cc blood, $P < 0.05$). Thus, AMI causes a significant increase in circulating ECFC in affected pigs.

Next, we questioned whether the distribution of ECFC proliferative potential would be altered following ischemia in these different test blood samples. There was no statistical difference in the percentage of single cells dividing at least once between the groups at baseline, post infarction and 7 days later (Figure V.5A-B). Strikingly, the percentage of single cells giving rise to colonies of more than 2000 cells (HPP-ECFCs) dramatically increased in the blood following AMI compared to the sham control blood samples ($38.4 \pm 5.8\%$ vs. $0.4 \pm 0.4\%$, $P < 0.05$) (Figure V.5C) and also to baseline blood samples ($38.4 \pm 5.8\%$ vs. $2.4 \pm 2.4\%$, $P < 0.05$) (Figure V.5D). On the other hand, the percentage of single cells that formed colonies containing 51-500 cells remarkably decreased following infarction compared to sham control samples ($29.2 \pm 2.2\%$ vs. $76.2 \pm 4.5\%$, $P < 0.05$) (Figure V.5C). The percentage of endothelial clusters (endothelial colony with 2-50 cells) present in the blood after AMI was also reduced compared to baseline (Figure V.5D). Finally, the percentage of HPP-ECFC in the blood samples drawn 7 days after AMI remained 2-fold greater than that measured at baseline, but this trend did not reach significance. In contrast, the distribution of ECFC in sham control animals was not disturbed from baseline following instrumentation (Figure V.5E).

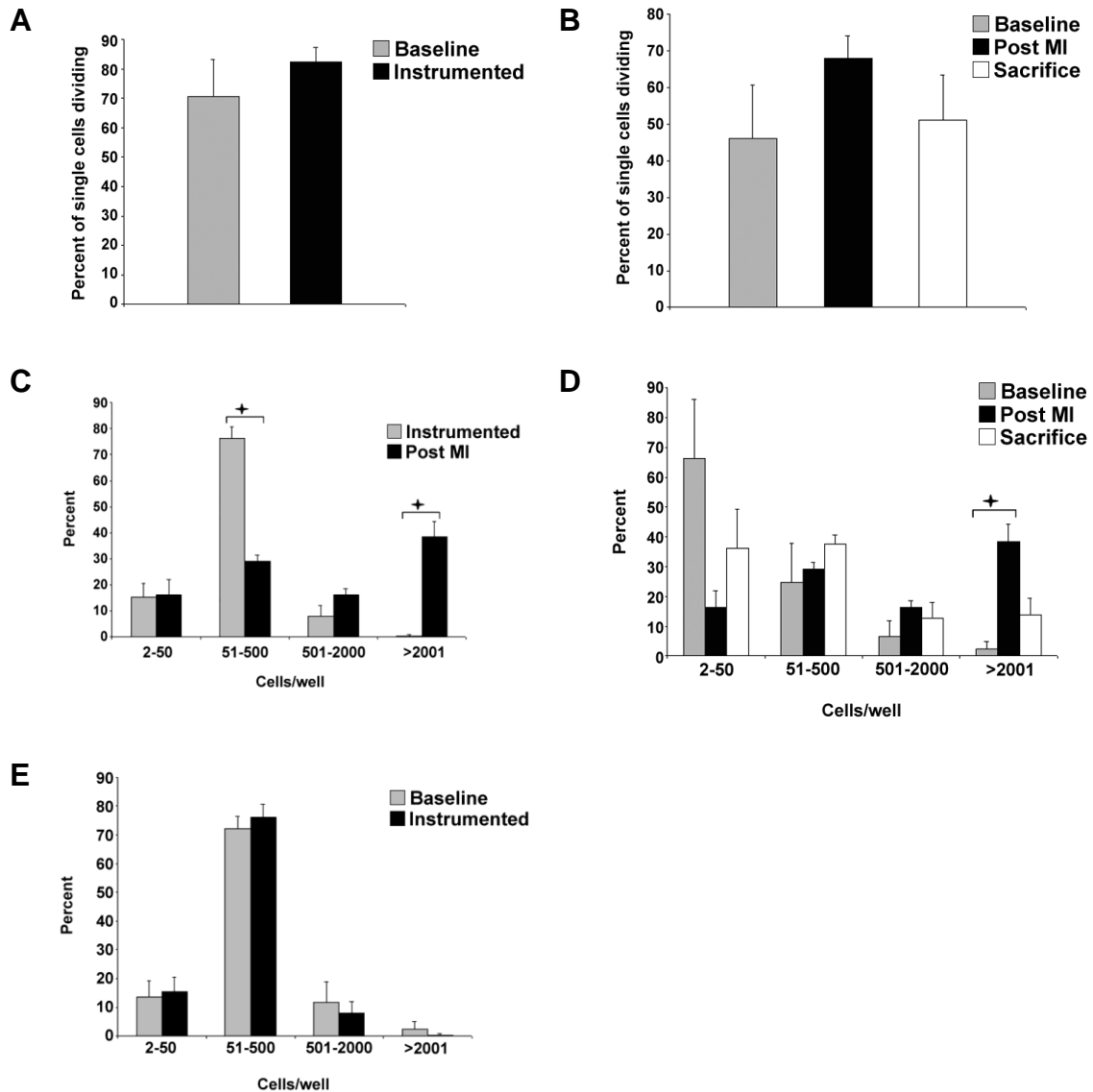


Figure V.5 Quantitation of the clonogenic and proliferative potential of single endothelial cells derived from PB ECFCs in the swine undergoing experimental AMI. (A-B) The percentage of single PB ECFC-derived ECs dividing at least once after 14 days culture in the sham control group (n=3) (A) and in the experimental group (n=5) (B) when isolated at the indicated timepoints. (C) The comparison of the distribution of different sized colonies from a single PB ECFC-derived ECs in an individual well after 14 days of culture between Post MI and sham control, each isolated at 45 min following

coronary cannulation (D-E). The harvest time-dependence of the distribution of different sized colonies from single PB ECFC-derived ECs are shown for individual wells in the experimental group (D) and in the control group (E). Observations were made after 14 days of culture. * $P < 0.05$ by nonparametric (Wilcoxon or Mann-Whitney) test.

Discussion

This is the first study to define a hierarchy of ECFCs circulating in the bloodstream or present in blood vessels in swine. Using a single cell clonogenic assay, ECFCs could be resolved based on their proliferative potential. Further, we find that the number and distribution of circulating ECFCs is altered in experimental pigs within 45 minutes following onset of AMI.

Previously we reported a hierarchy of ECFCs exists in human blood and blood vessels (Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004). The ECFCs were present in higher frequency in human umbilical cord blood than in adult peripheral blood and the distribution of ECFCs was weighted toward more HPP-ECFC in the cord blood samples. The human ECFCs displayed a variety of cell surface proteins previously identified on human blood vessel endothelium and formed blood vessels when implanted in collagen gels into immunodeficient mice (Yoder et al 2007). Thus, human ECFCs display properties consistent with stem/progenitor cells for the endothelial lineage. Using the same strategy, a hierarchy of ECFCs was identified in porcine circulating blood and blood vessels in the current study.

Growing evidence suggests that changes in the number of EPCs and CECs correlate with the risk of developing certain human clinical disorders. A decrease in number or impaired function of EPCs has been observed in patients with diabetes, heart failure and coronary artery diseases (Chen et al 2004, Eizawa et

al 2004, Loomans et al 2004, Tepper et al 2002). Moreover, the number and function of EPCs inversely correlates with the occurrence of cardiovascular events and death from cardiovascular causes (Hill et al 2003, Schmidt-Lucke et al 2005, Vasa et al 2001, Werner et al 2005). Conversely, elevated numbers of CECs have been demonstrated in patients with a variety of clinical disorders such as sickle cell crisis, septic shock, acute myocardial infarction, acute ischemic stroke and coronary artery disease (Güven et al 2006, Mutunga et al 2001, Nadar et al 2005, Solovey et al 1997, Wang et al 2005). Consistent with these findings, late outgrowth ECFCs are increased in the bloodstream of patients with severe coronary artery disease (Güven et al 2006). Based on these human clinical observations, we have hypothesized that the change in circulating ECFC count and/or distribution could also be a surrogate biological marker for vascular function and cumulative cardiovascular risk and be utilized to predict the outcomes of vascular diseases.

In this study, the frequency of circulating ECFCs in the blood of normal swine was quite low at baseline, but quickly increased with the induction of cardiac injury. Additionally, our study, based on a single cell clonogenic assay, provides evidence that an AMI could affect the proliferative distribution of ECFCs, specifically stimulating more HPP-ECFC emergence in the circulating blood. These results suggest that an AMI induces either an acute release of the ECFC from the damaged area or release of a mobilizing molecule that recruits the HPP-ECFC into the circulation. Of interest, Güven et al (Güven et al 2006) recently

reported that the concentration of late outgrowth endothelial cells is increased in those patients with the most severe coronary artery disease, and those patients selected for a revascularization procedure displayed a significantly higher number of circulating late outgrowth endothelial colony forming cells than patients who did not require a revascularization procedure; the mechanisms underlying this finding are not yet known. Multiple growth factors and cytokines are known to be released in the setting of AMI or exercise-induced ischemia, but their significance for ECFC dynamics is not known. The establishment here of an animal model with the property of robust and predictable ECFC mobilization forms the background for future studies directed towards uncovering potential molecular mechanisms for this mobilization. A comparison of factors circulating at baseline and during ischemia, and selective manipulation of these factors should provide insight into these mechanisms as well as potential future therapeutic targets.

Endothelial cell proliferation in normal, mature vessels in most mammals is reported to be extremely low. In some experimental animals, such as rats, guinea pigs and dogs tritiated thymidine labeling studies demonstrated that 0.1-3.0% of endothelial cells proliferate daily (Caplan & Schwartz 1973, Schwartz & Benditt 1973, Schwartz & Benditt 1976). In contrast to this slow turnover of endothelial cells in normal vessels, in vitro plating of swine aortic endothelial cells was associated with rapid proliferation, a finding consistent with numerous studies using human or bovine vessel-derived endothelial cells (Bompais et al 2004,

Cajero-Juarez et al 2002, Ingram et al 2005b, Ryan et al 1978). Furthermore, a complete hierarchy of ECFCs was present in the porcine vessel wall-derived endothelial cells, similar to that observed in circulating blood and consistent with that published for human blood and blood vessel-derived endothelial cells (Ingram et al 2005a, Ingram et al 2005b, Ingram et al 2004). These data suggest that some of the endothelial cells residing in the blood vessel intimal layer under normal circumstances are quiescent but retain proliferative potential that is displayed upon releasing the cells from contact inhibition and plating the cells in vitro. Future studies will be required to determine whether the source of inducible ECFC mobilization in porcine and human subjects is derived from the vascular endothelial intima of the host vessels or from extravascular marrow niches.

In this study we compared the expression of a number of cell surface antigens in porcine hematopoietic MNCs and aortic and blood ECFC-derived ECs. Both types of endothelial cells showed similar expression pattern of these surface proteins, which was different from that of blood MNCs (Figure V.3C).

Interestingly, these porcine ECFC-derived ECs expressed some surface antigens that were reported to be present in hematopoietic cells, such as the pan-tissue marker (granulocytes, monocytes and lymphocytes), whereas the common leukocyte surface marker CD45 and SLA-DR were not detected. Available evidence suggests that during embryonic development the hematopoietic stem cells (HSCs) and EPCs are derived from a common precursor, the hemangioblast, and then differentiate into different lineages and play distinct

functions (Baron 2003, Cogle & Scott 2004). This fact has led some investigators to postulate that circulating blood cells isolated from human and murine bone marrow transplant recipients or following development of leukemia provide evidence of a hemangioblast precursor for the hematopoietic and endothelial lineages in these adult subjects. Other than the pan-tissue marker, we saw no other evidence of cell surface antigen co-expression of markers between the hematopoietic and ECFC in this study.

In conclusion, we report that ECFCs are present in normal circulating swine blood and comprise a proportion of the endothelial cells that represent the aortic endothelial intima. Though the circulating ECFCs are rare, some of these cells possess remarkable proliferative potential, also displayed by some of the ECFCs that reside as aortic endothelial cells. The onset of acute myocardial ischemia leads to prompt changes in circulating ECFC number and the selective mobilization of highly proliferative subpopulations. Identification of populations of circulating and resident vascular endothelial cells with such potent proliferative potential may improve our understanding of the cellular mechanisms of angiogenesis and permit new strategies to manipulate these cells to protect the vascular endothelium from injury and disease.

CHAPTER VI

Summary and Perspectives

Circulating EPCs have been identified for more than a decade and have been indicated to contribute to neovascularization via postnatal vasculogenesis.

However, the definition of an EPC remains controversial. Our lab has successively isolated and identified ECFCs in circulating blood and blood vessels in human and other species. Based on the strictest definition of an EPC as a cell that 1) displays the ability to produce endothelial progeny that form endothelial tubes in vitro and 2) contributes to the functional endothelial lining of injured vascular structure via angiogenesis and/or vasculogenesis in vivo, to date, only ECFCs fit the definition of true EPCs and display all the activities described on these chapters. Furthermore, we have extended this definition and identified the stem/progenitor cells for the corneal endothelial lineage.

Corneal endothelium is formed by migration and proliferation of neural crest-derived mesenchymal cells and plays a barrier-bump function to maintain corneal clarity. Unfortunately, corneal ECs fail to proliferate in vivo even in response to corneal trauma or diseases which causes cell loss and eventually leads to the loss of vision. However, they do display replicative capacity in vitro. In the present study, using a single-cell clonogenic assay, we are able to first demonstrate that not all corneal ECs display the same ability to proliferate at a clonal level. We have defined a hierarchy of ECFCs in bovine corneal endothelium based on their proliferative potential, which is quite similar to bovine

vascular ECs resident in aorta, coronary artery, and pulmonary artery and is consistent with circulating and resident vascular ECs in adult human subjects. Thus, this study provides a new conceptual framework for defining corneal EPC potential and contributes to identify corneal endothelial precursor which is currently controversial. Additionally, HPP-ECFCs in corneal endothelium with their vast proliferative potential may represent a novel cell source for reconstituting corneal endothelium and be of great benefit to corneal transplantation.

Although human cord blood derived ECFCs are able to de novo form vessels, molecular mechanisms underlying the roles of ECFCs in neoangiogenesis remain largely unknown. Several fundamental biological questions on EPCs' mobilization, homing, differentiation, lumenization and more are pending answered. One of them that whether circulating ECFCs can be specified to an arterial or venous fate has so far not been addressed. Thus, we hypothesized that human cord blood derived ECFCs are not committed to either an arterial or venous fate and are able to be differentiated into arterial ECs when given appropriate stimuli. In the present study, we first demonstrate that human cord blood derived ECFCs heterogeneously express molecules that normally are restricted to ECs resident in arteries and veins. In vitro, exposure to Notch ligand Dll1 is sufficient to induce an arterial gene expression pattern in ECFCs. However, the in vitro Dll1-primed ECFCs, when implanted, failed to display significant weighting toward an arterial EC phenotype or function in vivo. Therefore, the in vivo formation of human cord

blood ECFCs derived, long-lasting, functional vessels with an arterial identity needs further investigated probably by manipulating implant microenvironments. Nevertheless, this study contributes to the understanding of possible mechanisms involved in AV differentiation in vitro and postnatal arteriogenesis in humans and provides novel insights into the development of cellular therapeutic strategies for patients with coronary and peripheral arterial diseases.

There is growing evidence suggesting that the concentration of circulating ECs is close related to the adverse progression of cardiovascular disorders. Based on human clinical observations, we hypothesized that the alteration in circulating ECFC count and/or proliferative distribution could be a surrogate biological marker for vascular function and cumulative cardiovascular risk and be utilized to predict the outcomes of vascular diseases. Pigs are a favored species to examine the pathophysiology of coronary artery injury and acute myocardial ischemia (AMI). By using this animal model, we have demonstrated that the number of circulating ECFCs in the bloodstream in healthy swine is quite rare. The onset of AMI leads to a prompt increase in circulating ECFC number and a selective mobilization of HPP-ECFCs. The exact role of the mobilized HPP-ECFCs in homing and participation in repair of the ischemic tissue remains unknown. Moreover, multiple growth factors and cytokines have been known to be released in the setting of AMI. Future, a comparison of factors circulating before, during and after ischemia, and selective manipulation of these factors would provide insights into these uncovering potential molecular mechanisms for

the mobilization of HPP-ECFCs. The identification and characterization of HPP-ECFC may improve our understanding of the cellular mechanisms of neoangiogenesis and permit new strategies to manipulate these cells to protect the vascular endothelium from injury and disease.

Based on their multiple therapeutic potentials, human ECFCs have become an attractive target for novel vascular regenerative therapies. However, the study of ECFCs is hampered by several challenges in culturing cells. Current protocols for in vitro expansion of ECFCs mostly depend on the presence of FBS or FCS in the culture medium which may disturb chromosome stability, modify gene expression and thereafter affect cell properties and functions, and have risky potentials of transmitting infectious agents and inducing immune reactions when future used in a transplantation setting. According to these regards, we have developed a novel formula of endothelial cell culture medium that is free of FBS and is supplemented with 1.5% HCP for expansion of human cord blood ECFCs in vitro. In creating this medium, we have also demonstrated that ECFCs grown on this medium retain their phenotype and function, as well as their proliferative and neoangiogenic properties. This work contributes to the development of a complete serum-free culture system for ECFCs that we and others believe will be required for future use in human cell therapeutics.

In summary, these studies contribute to an improved understanding of ECFCs and their roles in postnatal vascularization and suggest several possible therapeutic applications of ECFCs.

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CURRICULUM VITAE

Lan Huang

Education

2009	Ph.D. Indiana University, IN
2003	M.S. Xiamen University, China
2000	B.A. Xiamen University, China

Fellowships and Awards

2009	Travel award, the 7th Midwest Blood Group Meeting
2008	AHA Predoctoral Fellowship (0810062Z), "Are circulating endothelial progenitor cells committed to an arterial or venous fate?"
2007	Graduate fellowship in 'Translational Research', Indiana University School of Medicine
2007	Travel award, Indiana University School of Medicine
2007	Travel award, the 6th Annual Gene Therapy Symposium for Heart, Lung, and Blood Diseases
2000	Outstanding undergraduate research project, Xiamen University, China

Professional Affiliation

North America Vascular Biology Organization

International Society of Experimental Hematology

Presentations

- 2009 Dose-dependent effects of VEGF165 and the Notch ligand Delta1 on ex vivo differentiation of human cord blood derived endothelial colony forming cells (ECFCs). 7th Midwest Blood Group Meeting, Cincinnati Ohio (Oral)
- 2008 Endothelial colony forming cells (ECFCs) isolated from the rhesus monkey are phenotypically and functionally similar to human circulating ECFCs. ISEH 37th Annual Scientific Meeting Featuring the 6th International Neonatal Hematology and Immunology Meeting, Boston, MA (Poster)
- 2007 A hierarchy of endothelial colony forming cell proliferative activity is displayed by bovine corneal endothelial cells. 5th Midwest Blood Group Meeting, Cincinnati Ohio (Poster)
- 2007 A hierarchy of endothelial colony forming cell proliferative activity is displayed by bovine corneal endothelial cells. 48th Annual Midwest Society for Pediatric Research Scientific Meeting, Indianapolis, IN (Poster)
- 2007 Endothelial colony forming cells (ECFCs) isolated from the rhesus monkey are phenotypically and functionally similar to human circulating ECFCs. 6th Annual Gene Therapy Symposium for Heart, Lung, and Blood Diseases, Sonoma, CA (Oral)
- 2006 Alteration of the distribution of endothelial colony forming cells (ECFCs) in a swine model of acute myocardial ischemia (AMI).

Gordon Research Conference in Endothelial Cell Phenotypes in Health & Disease, Biddeford, ME (Poster)

- 2006 Alteration of the distribution of endothelial colony forming cells (ECFCs) in a swine model of acute myocardial ischemia (AMI).
47th Annual Midwest Society for Pediatric Research Scientific Meeting, Indianapolis, IN (Oral)

Publications

1. Lung microvascular endothelium is enriched with progenitor cells that exhibit rapid vasculogenic capacity. Alvarez DF, **Huang L**, King JA, Yoder MC, Stevens T. *Am J Physiol Lung Cell Mol Physiol*. 2008 Mar;294(3):L419-30.
2. Regulatory role for nucleosome assembly protein-1 in the proliferative and vasculogenic phenotype of pulmonary endothelium. Clark J, Alvarez DF, Alexeyev MF, King JA, **Huang L**, Yoder MC, Stevens T. *Am J Physiol Lung Cell Mol Physiol*. 2008 Mar;294(3):L431-9.
3. Circulating angiogenic precursors in idiopathic pulmonary arterial hypertension. Asosingh K, Aldred MA, Vasanji A, Drazba J, Sharp J, Farver C, Comhair SA, Xu W, Licina L, **Huang L**, Anand-Apte B, Yoder MC, Tudor RM, Erzurum SC. *Am J Pathol*. 2008 Mar;172(3):615-27.
4. Acute Myocardial Infarction in swine rapidly and selectively releases highly proliferative Endothelial Colony Forming Cells (ECFCs) into circulation. **Huang L**, Hou DM, Thompson MA, Baysden SE, Shelley WC, Ingram DA, March KL, Yoder MC. *Cell Transplantation*. 2007;16(9):887-97.

5. VEGF and IHH rescue definitive hematopoiesis in Gata-4 and Gata-6 deficient murine embryoid bodies. Pierre M, Yoshimoto M, **Huang L**, Yoder MC. Exp Hematol. 2009;37(9):1038-53.
6. A hierarchy of endothelial colony forming cell activity is displayed by bovine corneal endothelial cells. **Huang L**, Harkenrider M, Baysden SE, Thompson MA, Zeng PY , Bonanno JA, Ingram DA, Yoder MC (in preparation)
7. Dose-dependent effects of VEGF165 and the Notch ligand Delta1 on ex vivo and in vivo differentiation of human cord blood derived endothelial colony forming cells (ECFCs). **Huang L**, Critser PJ, Mallett CP, Yoder MC (in preparation)